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THE LESIONS IN RABBITS EXPERIMENTALLY INFECTED BY A VIRUS ENCOUNTERED IN THE ATTEMPTED TRANSMISSION OF VARICELLA.

By THOMAS M. RIVERS, M.D., and WILLIAM S. TILLETT, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

Plates 6 to 9.

(Received for publication, May 16, 1924.)

During attempts to infect rabbits with the virus of varicella, an active transmissible agent has been encountered which partakes of the characters of the so called filterable viruses. The methods employed, the behavior of the virus in normal and immune rabbits, and the results of attempts to identify the virus have been described in previous papers.[1,2] Early in the work the method employed[1,2] in procuring the virus and the gross and microscopic appearances of the lesions produced by the virus in the testicles, skin, and corneas of rabbits led to the inference that this virus was not improbably the etiological agent of chicken-pox. No proof was forthcoming, however, from immunity studies in rabbits and in man. On the other hand, evidence was obtained that there had been encountered a hitherto unrecognized active transmissible agent which would be of especial interest to investigators working with filterable viruses in rabbits.

The exact source and nature of the virus and its relation to human and rabbit diseases remain to be determined. Nevertheless, the lesions produced in rabbits by the virus and the intracellular changes found in these lesions resemble so closely those seen in varicella[3] and herpes zoster that it seems desirable to describe them without further delay.

[1] Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1923, xxxviii, 673.
[2] Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1924, xxxix, 777.
[3] Tyzzer, E. E., *J. Med. Research*, 1905–06, xiv, 361.

These observations should be of special interest since workers[4-6] have reported that specific lesions occur in the scarified corneas of rabbits inoculated with vesicle fluid from varicella and herpes zoster patients.

Method.

Tissues for examination were removed[7] 3 to 6 days following inoculations with the virus. After many fixatives and stains had been tried, Zenker's fluid with 5 per cent acetic acid, and methylene blue-eosin and Giemsa stains were found entirely satisfactory. When no mention is made of the fixative and stain employed, it will be understood that Zenker's fluid and methylene blue-eosin were used.

Testicles.

Gross Pathology.—The gross pathological changes occurring in the testicles when large amounts of testicular emulsions containing the virus were injected had of necessity to be disregarded. When small quantities of the supernatant fluid from centrifuged testicular emulsions containing active virus were inoculated into the testicles of normal young adult rabbits, significant gross changes also occurred. On the 3rd to 6th day after the inoculation, the testicles became swollen, tense, and tender. Often the scrotum was red and edematous. When the testicles were removed, they were red, swollen, and edematous and the surface was covered with yellowish spots. Upon sectioning the testicles the swollen condition was more evident as the parenchyma bulged out from the restraining capsule. The gross picture was one of acute inflammation.

Microscopic Pathology.—Testicles inoculated with large amounts of the virus showed diffuse lesions involving the whole testicle and consisting of marked degeneration and dilatation of the tubules, engorgement of the blood vessels, and an increase in the cells and fluid in the interstitial tissue (Fig. 1). The cellular infiltration consisted of endothelial leucocytes (Fig. 2), plasma cells, lymphocytes,

[4] Lipschütz, B., *Arch. Dermatol. u. Syph., Orig.*, 1921, cxxxvi, 428.
[5] Bertarelli, E., *Centr. Bakt., 1. Abt., Orig.*, 1909, l, 181.
[6] Gins, H. A., *Z. Hyg. u. Infectionskrankh.*, 1918, lxxxvi, 299.
[7] All operations were performed under ether anesthesia.

and in some instances polymorphonuclear leucocytes. The most characteristic finding was the decided increase in the endothelial leucocytes (Fig. 2), many of which contained two nuclei and nuclear inclusions (Fig. 11). When small amounts of virus were injected into the testicles, discrete lesions were observed in the interstitial tissue with the same cellular changes described above.

Skin.

Lesions Following the Inoculation of Virus on the Scarified Skin.

Gross Pathology.—4 to 6 days after inoculation of the virus on the scarified skin of rabbits discrete lesions appeared in the form of small, superficial, red macules and papules. These persisted for 3 or 4 days, and then disappeared without scar formation. The discrete lesions might be overlooked but when confluent ones occurred along the lines of scarification they were not so likely to be missed. The confluent lesions exhibited more swelling of the tissues and persisted longer than the discrete ones. No definite vesicle formation was observed in any of the lesions.

Microscopic Pathology.—The epidermis was thickened. No definite vesicles were observed. The epidermal cells were swollen and stained a pale pink. The corium was edematous and exhibited a marked cellular infiltration consisting of endothelial leucocytes, plasma cells, lymphocytes, and at times polymorphonuclear leucocytes (Figs. 3 and 4). In some of the endothelial leucocytes nuclear inclusions were seen which resembled those found in testicular tissue.

Lesions Following the Intradermal Inoculation of Virus.

Gross Pathology.—Following the intradermal inoculation of the virus nothing except the effects of trauma was observed for 3 or 4 days. Then the skin at the site of the injection of the virus became red and raised. The reaction at these points increased in size and intensity till the 5th to the 7th day, when the central portion of the inoculated area was often very dark. Extending into the skin around the dark central area was a zone of less intense erythema 2 to 4 cm. in width. The lesions gradually subsided and in a fortnight disappeared without scar formation.

Microscopic Pathology.—The surface of the skin was often covered by a layer of desquamated cells of various kinds. Beneath this layer the epidermis was seen. It was not always intact, however, breaks in its continuity being frequently observed. The epidermal cells were undergoing degeneration, stained poorly, and a few of them contained nuclear inclusions. In the upper part of the corium there was considerable generalized edema with localized collections of fluid suggestive of vesicles (Fig. 5). The cellular infiltration in the corium was marked and consisted of endothelial leucocytes, plasma cells, lymphocytes, and polymorphonuclear leucocytes. The endothelial leucocytes were numerous and many of them contained nuclear inclusions.

Discrete Lesions in the Shaved Skin Following Intratesticular Inoculation of Virus.

Gross Pathology.—In some of the rabbits inoculated only in the testicles areas of skin were shaved. A diffuse or macular erythema which disappeared in 2 or 3 days followed merely this shaving alone. In addition to the lesions thus induced, discrete red macules and papules frequently appeared in the skin 5 to 11 days after the intratesticular inoculations, persisted 3 to 7 days, and disappeared without scar formation. At times a zone of erythema less intense than that of the papules surrounded each lesion. These discrete lesions we have interpreted as the result of a generalized infection with the virus.

Microscopic Pathology.—The epidermis was thickened (Fig. 6). No vesicles were observed. The epidermal cells were swollen and stained poorly. The upper part of the corium was edematous. The cellular infiltration of the corium, consisting largely of endothelial leucocytes and plasma cells, was frequently most marked around small blood vessels (Fig. 6). Many of the endothelial leucocytes were swollen and an occasional one contained nuclear inclusions. Lymphocytes and polymorphonuclear leucocytes, although present at times, were not numerous.

Lesions in Scarified Corneas Inoculated with Virus.

Gross Pathology.—Scarified corneas (cocaine anesthesia was always used) inoculated with virus showed a reaction which was absent

in the controls. The reaction was noticeable in 3 or 4 days, persisted 4 or 5 days, and disappeared rapidly without permanent injury to the eye unless a secondary infection occurred. The corneas were roughened and slightly opaque, especially along the lines of scarification. Increased lacrimation, photophobia, and injection of the blood vessels around the corneas were usually observed.

Microscopic Pathology.—The defects in uninoculated scarified corneas were repaired rapidly with young corneal cells which appeared normal in every respect (Fig. 7). In contrast to this, eyes inoculated with virus exhibited definite lesions in the young corneal cells filling the defects in the corneas produced by scarification. In these lesions very few cells were seen which were not of epithelial origin. Therefore, corneal lesions were most satisfactory for the study of the characteristic intracellular changes which are frequently seen in cells injured by the virus. These cells were swollen and often stained poorly, some had two to four nuclei (Fig. 9), and many had nuclear inclusions (Figs. 8 to 10). Only a few cells were involved in the early lesions. Later when more cells had been injured and the degeneration of these cells had progressed, it was evident that a small vesicle was in process of formation (Fig. 9). Finally, as the cells degenerated more and more, definite vesicles were formed, filled with fluid and the remains of degenerated cells. Around the vesicles many of the cells were in various stages of degeneration and some contained nuclear inclusions (Fig. 10). The early stages of vesicle formation were frequently seen. Well developed vesicles, however, similar to the one shown in Fig. 10 were not observed often. The vesicles were delicate and many of them may have been destroyed in the process of making sections. Only a slight reaction occurred beneath the corneal cells and this usually consisted of a mild infiltration of endothelial leucocytes some of which had nuclear inclusions.

Nuclear Inclusions.

Nuclear inclusions (Figs. 11 and 12) were numerous in endothelial leucocytes in inoculated testicles (Fig. 2) and in corneal cells of inoculated eyes (Figs. 8 and 10). They were less numerous in the endothelial leucocytes of skin lesions and were rarely observed in

epidermal cells. In early lesions these inclusions occurred in the nuclei of otherwise normal looking cells and appeared as small pink dots surrounded by a clear zone, the so called halo. As the lesions became older the inclusions increased in size and were still surrounded by a halo. The uninvolved nuclear material seemed to draw away from the inclusions which finally occupied most of the nuclear space. The remaining uninvolved nuclear material often with the nucleolus was then compressed against the normally thin nuclear membrane and the two together stained a dark blue giving the appearance of an unusually heavy nuclear membrane. In the meantime the cell became swollen and finally death ensued as indicated by the whole cell taking a pink stain. In properly stained sections all the inclusions were pink, in contrast to the blue stain usually taken by nuclear material; the small ones stained homogeneously, the larger ones, however, frequently had the appearance of being an aggregation of the smaller ones.

DISCUSSION.

Local infiltration of the tissues with endothelial leucocytes, swelling of the involved epithelial cells, and the presence of nuclear inclusions in both the endothelial leucocytes and the epithelial cells were the characteristic pathological changes observed in rabbits experimentally inoculated with the virus under investigation. While characteristic of the action of the virus, these pathological changes cannot be considered as unique with it, since similar changes have been found in varicella, herpes zoster, symptomatic herpes, and other diseases resulting from infection with filterable viruses. The point of main interest in the present findings lies in the fact that this well known type of lesion, associated in the general mind with the action of a certain group of viruses, is produced by a hitherto unrecognized entity which partakes of the general characters of the group.

CONCLUSION.

The pathological changes produced by the virus under investigation are similar to those deemed characteristic of a certain well known group of filterable viruses.

EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. Lesion in the testicles of a rabbit, 4th day after intratesticular inoculation, showing the cellular infiltration in the interstitial tissue and the injury to the tubules. × 105.

FIG. 2. Higher magnification of section in Fig. 1, showing nuclear inclusions in endothelial leucocytes. × 1,500.

PLATE 7.

FIG. 3. Normal rabbit skin; the epidermis is thin, the corium not very cellular. × 105.

FIG. 4. Skin 4 days after dermal inoculation; the epidermis is thickened and there is an exudation of serum and cells into the corium. × 105.

FIG. 5. Skin 5 days after intradermal inoculation. There is a layer of desquamated cells on the surface. The epidermis is injured and its continuity is broken in places. Exudation of cells and serum into the corium is very marked. × 120.

FIG. 6. Lesion in the shaved skin of a rabbit inoculated only in the testicles; epidermis thickened, exudation of serum and cells into the corium. The cellular exudation consists principally of endothelial leucocytes and is most marked around small blood vessels. × 105.

PLATE 8.

FIG. 7. Lesion produced in a normal cornea by scarification alone. The eye was removed under ether 4 days after the scarification. × 300.

FIG. 8. Early lesion in an inoculated cornea; degeneration of the corneal cells, some of which contain nuclear inclusions. × 300.

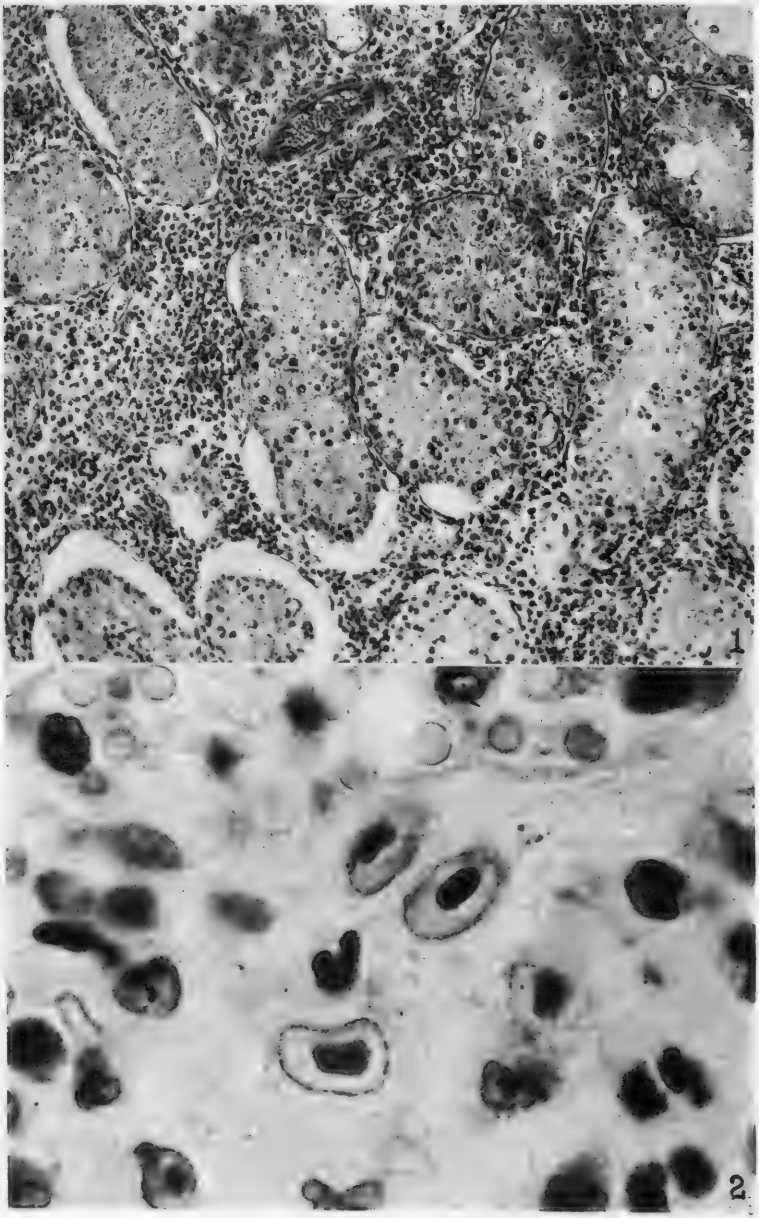
FIG. 9. Early vesicle formation in the cornea of an inoculated eye; degenerating cells with nuclear inclusions, giant cell with four nuclei. × 280.

FIG. 10. Vesicle in the cornea. Many of the cells around the vesicle contain nuclear inclusions. × 300.

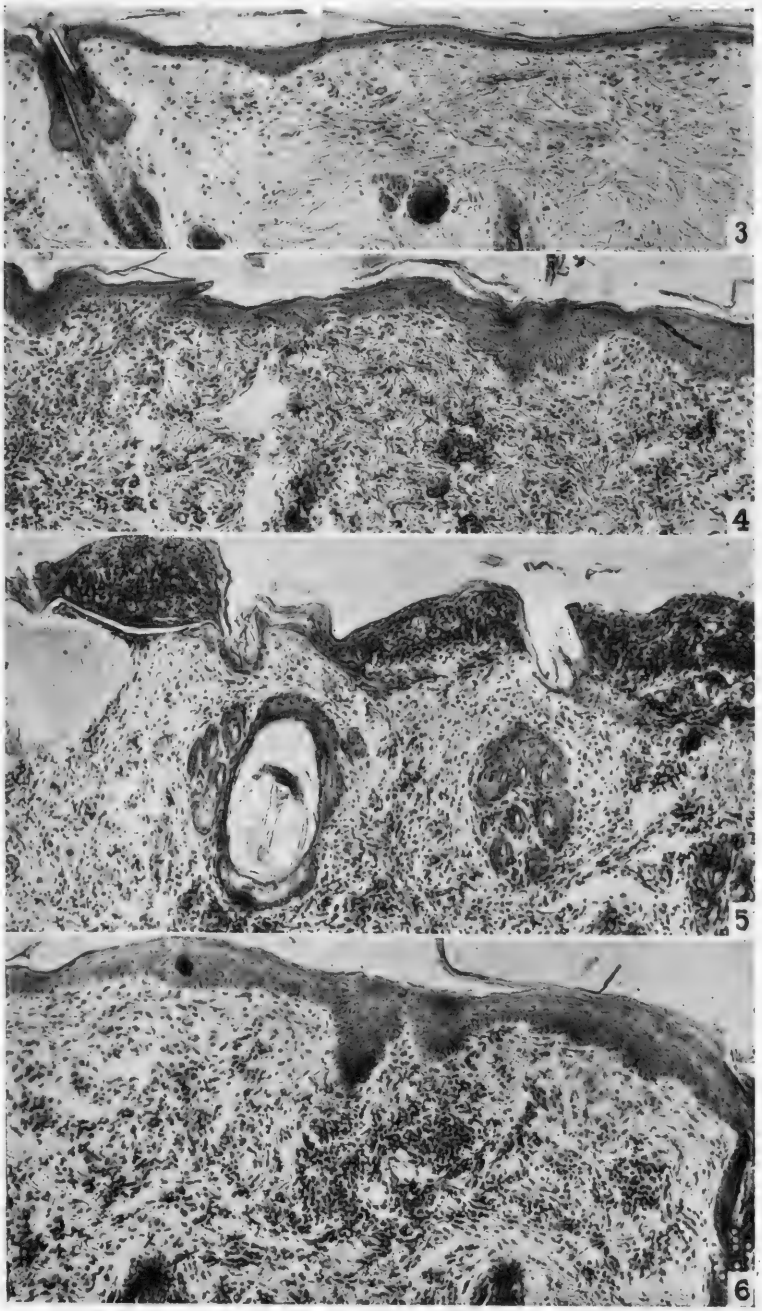
PLATE 9.

FIG. 11. Nuclear inclusions in endothelial leucocytes of an inoculated testicle. One cell has two nuclei both of which have inclusions. Normal cell for comparison. Eosin-methylene blue. × 1,500.

FIG. 12. Nuclear inclusions in corneal cells of an inoculated eye. The small inclusions stain homogeneously, whereas the large inclusions do not always seem to be homogeneous. Each inclusion is surrounded by a halo. Normal cell for comparison. Giemsa. × 1,500.



(Rivers and Tillett: Attempted transmission of varicella.)



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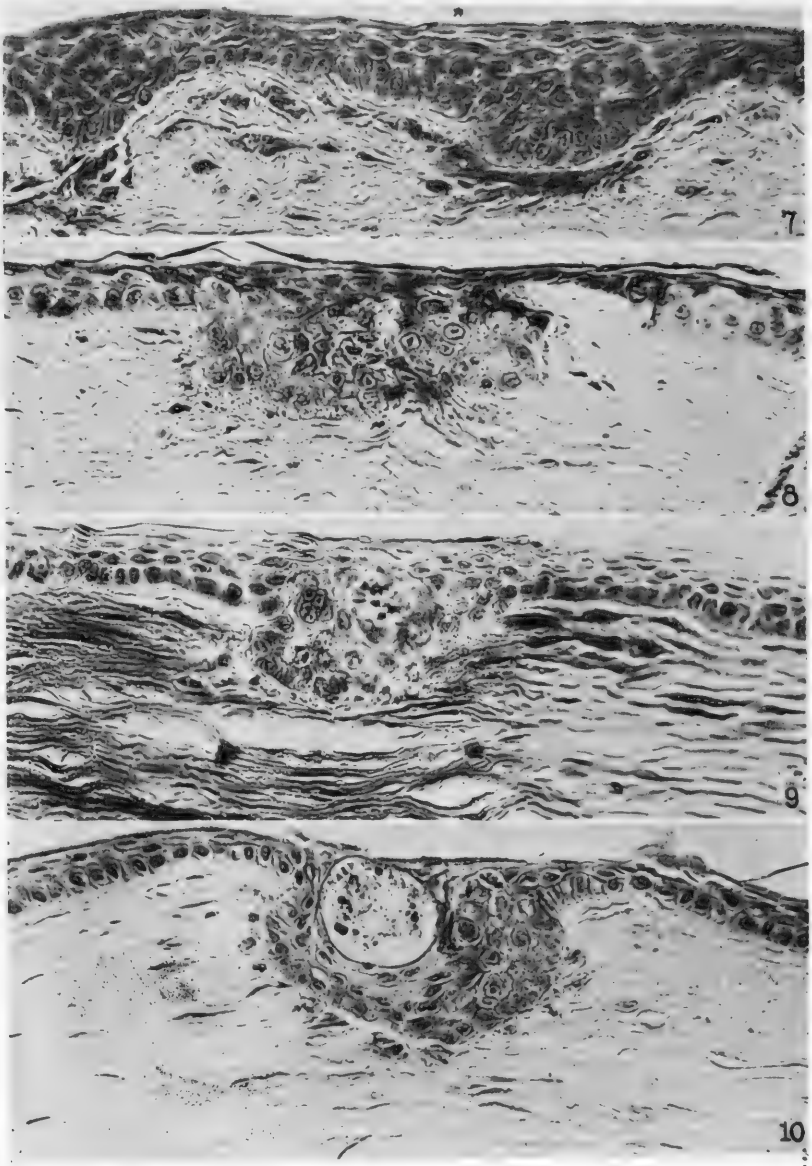

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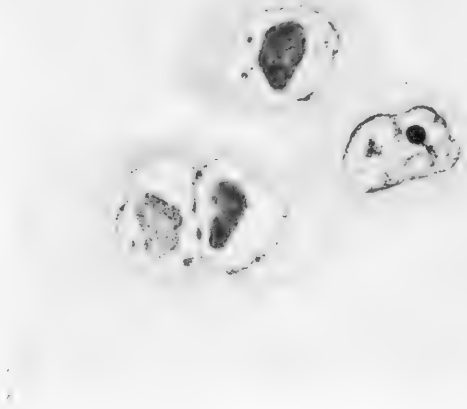

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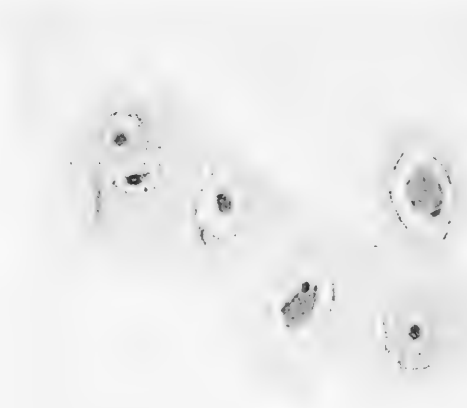

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(Rivers and Tillett: Attempted transmission of varicella.)

PARTIAL OCCLUSION OF THE PULMONARY AORTA AND INFERIOR VENA CAVA WITH THE METALLIC BAND. OBSERVATIONS ON CHANGES IN THE VESSEL WALL AND IN THE HEART.

By MONT R. REID, M.D.

(*From the Hunterian Laboratory for Experimental Surgery of the Johns Hopkins University, Baltimore.*)

PLATE 10.

(Received for publication, April 23, 1924.)

In 1916, some results of the application of metallic bands to the aorta of dogs were reported.[1] Later in the fall of the same year, constricting bands were applied to the common pulmonary artery, and, a year afterwards, marked constriction of the inferior vena cava was produced. The purpose of the present paper is to report the results of applying bands to the pulmonary artery and vena cava.

Under ether anesthesia constricting bands were placed on the common pulmonary artery of five dogs. Four of them died within a month as a result of distemper, intussusception, or injuries from fighting. No death was attributable to the effect of the constricting band. One dog (A 19 operated upon November 29, 1916) lived and was kept under observation for a period of $30\frac{1}{2}$ months. In no way did its general health appear to be affected.

At operation the diameter of the pulmonary artery at the site of application of the band was $13\frac{1}{2}$ mm.; the external diameter of the band, the ends of which overlapped markedly, was 10 mm.; the width of the band was 5 mm.

[1] Halsted, W. S., An experimental study of circumscribed dilation of an artery immediately distal to a partially occluding band, and its bearing on the dilation of the subclavian artery observed in certain cases of cervical rib, *J. Exp. Med.*, 1916, xxiv, 271. Reid, M. R., Partial occlusion of the aorta with the metallic band. Observations on blood pressures and changes in the arterial walls, *J. Exp. Med.*, 1916, xxiv, 287.

Frequent x-rays of the heart did not give any evidence of an increase in size of the cardiac shadow. June 18, 1917. The electrocardiographic report was: "Marked sinus arrhythmia, otherwise a normal mechanism. P-R interval measures 0.10 second. P wave of normal amplitude, measuring 1.6 mm. T wave of large amplitude in Leads II and III, measuring 5 mm. No evidence of right or left ventricular preponderance." A second report, October 10, 1917, was: "Abnormal form of T wave. Sinus arrhythmia." A loud systolic bruit could be heard over the entire chest immediately following the operation and this remained present until the animal was sacrificed. No murmur could be heard previous to operation.

June 19, 1919. Under ether anesthesia, the femoral arterial blood pressure measured 107 mm. of mercury; the femoral venous pressure, 6 cm. of water. The animal was sacrificed. The heart weighed 137 gm., which is heavier than normal for a dog weighing only 12½ pounds. The right ventricular wall measured 1 cm., the left 1½ cm. Grossly and histologically, both ventricular walls looked very much alike. The hypertrophy of the right ventricle was apparently unassociated with any cardiac dilatation (Fig. 1). The valves of the heart were normal.

The band had produced a marked constriction in the lumen of the artery (Fig. 1). Proximal to the band the artery was large (diameter 15 mm.) and its wall was definitely thickened; distal it was of normal size (diameter 10 mm.) but the vessel wall appeared thin. The band had cut through the pulmonary artery on the side next to the thoracic aorta. A strong repair of the wall had occurred outside the band. The arterial wall surrounded by the band was in surprisingly good condition when we consider that the band had been present for 2½ years; for it is our experience that in the case of the thoracic or abdominal aorta, the wall would long before have died. Histological sections showed normal elastic tissue crossing under the band. The elastic tissue in the proximal portion of the artery was markedly increased, while distal to the band, it seemed much reduced (Fig. 2).

In many animals we placed constricting bands around the vena cava. When after several months these bands were removed there had not occurred any demonstrable gross or histological changes in the vessel wall. The folds of the intima even had not become adherent.

SUMMARY.

In dogs a constricting metallic band does not affect the integrity of the wall of the vena cava, whereas it leads to a rapid death of the wall of the aorta, and, as evidenced by a single instance studied, to a much slower death of the pulmonary artery. In the instance, just mentioned, constriction of the common pulmonary artery by an aluminum band for a period of $2\frac{1}{2}$ years led to a right ventricular hypertrophy.

EXPLANATION OF PLATE 10.

FIG. 1. Result, after $2\frac{1}{2}$ years, of constricting band on the pulmonary artery. Observe the marked constriction caused by the band (*A*) and the enlargement of the right ventricle.

FIG. 2. A longitudinal section of the pulmonary artery through the site of the band. Note the hypertrophy of the elastic wall proximal, and its atrophy distal to the band. Weigert's elastic tissue stain.

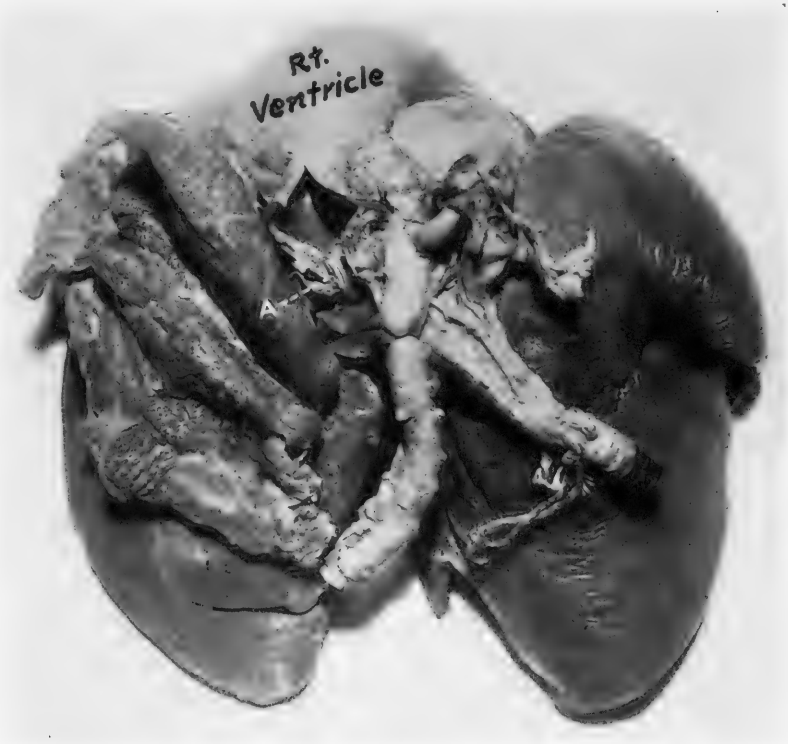

FIG. 1.

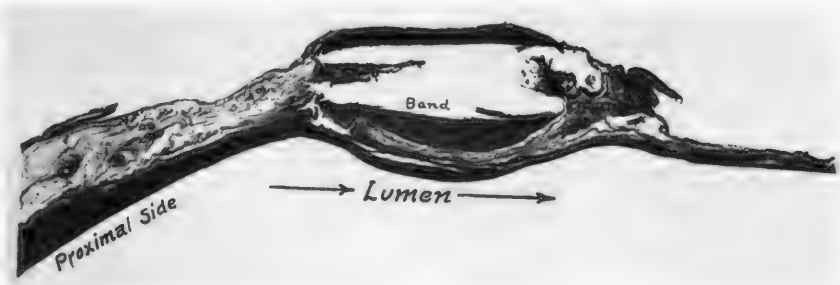

FIG. 2.

(Reid: Partial occlusion of pulmonary aorta.)

PARTIAL OCCLUSION OF THE AORTA WITH SILK SUTURES, AND COMPLETE OCCLUSION WITH FASCIAL PLUGS. THE EFFECT OF LIGATURES ON THE ARTERIAL WALL.

By MONT R. REID, M.D.

(*From the Hunterian Laboratory for Experimental Surgery of the Johns Hopkins University, Baltimore.*)

PLATES 11 AND 12.

(Received for publication, April 23, 1924.)

In experiments previously reported it has been observed that a partial or complete occlusion (either by a metallic band or ligature) of any large artery invariably results in a local death of the vessel wall.[1] This observation is of fundamental importance alike to the surgeon and pathologist, who have heretofore accepted the view that intimal surfaces of large arteries will unite when properly approximated.

Since it had been observed that a coarcting ligature or band always resulted in the death of the arterial wall, the following experiments were performed in order to find a method of producing a partial or complete occlusion which would not cause a necrosis.

1. Partial Occlusion by Means of Plication Sutures of Silk.

(*a*) *Method.*—The aorta of dogs was used for these experiments. Under ether the lumen of the vessel was reduced to about one-third its normal size by means of numerous mattress sutures of doubled medium silk (Fig. 1). The aorta was temporarily occluded above and below the point of constriction. Usually there was almost no bleeding from the needle punctures on releasing the temporary occlu-

[1] Reid, M. R., Partial occlusion of the aorta with the metallic band. Observations on blood pressures and changes in the arterial walls, *J. Exp. Med.*, 1916, xxiv, 287.

sion. In one instance a little piece of muscle was placed over the sutures in order to hasten complete hemostasis.

In this manner the thoracic aorta was partially occluded in two cases, and the abdominal aorta just above the inferior mesenteric artery in six cases.

(*b*) *Effect on the Lumen.*—The observations extended over a period of 2 to 8 months, and in each instance a partial occlusion persisted. As no means were employed to determine the narrowing of the lumen at the time of operation, it is impossible to know the alteration that occurred in it afterwards. It was, however, so slight that one may expect the lumen which is left at the time of plication to remain practically unchanged. In no instance did the vessel become completely occluded at the site of the operation. This observation is in accord with those of McLean,[2] who observed that sterile silk threads anchored in the lumen of large arteries did not lead to the formation of a thrombus while infected threads always did.

(*c*) *Effect of the Sutures on the Wall of the Vessel.*—Each individual mattress suture has the same effect on the portion of the vessel it includes as does the encircling metallic band or ligature, and like them leads to the death of the vessel wall. Tightly tied sutures cause marked destruction of and frequently cut through the vessel; more loosely tied sutures cause necrosis as evidenced by loss of staining reactions, although the necrosed tissue may remain for several months. About all of these mattress sutures there occurs a dense fibrous, connective tissue reaction, as well as in the path of a cutting suture (Fig. 2).

(*d*) *Effect on the Intima.*—Where the intimal surfaces are brought into apposition by the plicating sutures the intima may proliferate, and by bridging the crevice tend to restore the circular appearance of the lumen (Fig. 2).

(*e*) *Elastic Tissue.*—The regeneration of elastic tissue is said to be a very slow process. In these experiments, we have not seen any evidence of a fibrous segment becoming substituted by elastic tissue. In our studies with metallic bands it was observed in one instance that the fibrous wall which formed external to the band showed

[2] McLean, A., Thrombosis and embolism, *Surg., Gynec. and Obst.*, 1915, xx, 457.

at the end of 16 months a few strands of reformed elastic tissue. They seemed to appear as isolated islands of elastic tissue in the new wall rather than as a growth from the points where the elastic wall was destroyed by the band.

2. Total Occlusion with Plugs of Fascia.

(*a*) *Method.*—A small strip of fascia taken from the anterior sheath of the rectus muscle is made into the shape of a sphere with the aid of fine silk sutures. Through a small longitudinal slit made in a segment of the aorta, previously isolated by clamps, this fascial plug is introduced. By means of through and through sutures of silk it is anchored, either at the site of its introduction, or above or below it. The opening in the vessel is carefully closed with interrupted sutures of fine silk.

Six experiments were performed. The average length of time between the performance of the experiments, which were carried out under ether, and the recovery of the specimens was 6 months. Complete occlusion resulted in every instance, and there was neither infection nor hemorrhage.

(*b*) *Fate of the Fascial Plugs.*—These remain as viable tissue and do not become substituted by fibrous tissue. The transplanted fascia presents the appearance and staining characteristics of living tissue. Where the fascial plugs come in contact with the blood stream they become covered by intima, so that, when the obstruction is viewed either from above or below, it has the glistening appearance that is characteristic of the normal intima (Fig. 3).

(*c*) *Thrombosis and the Organization of a Thrombus.*—In two instances a small clot formed above and below the plug of fascia. A study of the organization of one of these clots has proven of great interest. Through breaks in the media of the aortic wall, the connective tissue is crowding its way toward the organized thrombus.

This observation and those on the organization of a necrosed vessel wall beneath metallic bands suggest that the organization of a thrombus within an intact vessel may be mainly from the media and adventitia rather than, as generally believed, from the intima (Figs. 4 and 5).

(*d*) *The Effect of a Plug on a Vessel Wall.*—The wall surrounding an occluding fascial plug does not die, though it atrophies. This atrophy extends for a considerable distance below the occlusion, whereas above the wall retains its normal size and thickness. The atrophy below a plug is similar to that observed below a partially occluding band. In the latter case, as presumably in the subclavian artery distal to a cervical rib, this atrophy may have some bearing on the development of an aneurism. We have not, however, seen an aneurismal dilatation below a complete occlusion, either by a band or fascial plug. The atrophy of the vessel wall surrounding a fascial plug does not lead to compensatory proliferation in the adventitia. Here, owing to lack of pressure, the tissues are not called upon to make the same protective changes as in the case of a cutting band or ligature (Fig. 6).

The silk sutures used to anchor fascial plugs are tied very loosely. They do, however, damage the vessel wall included by them.

DISCUSSION.

In 1914, Dr. Goodpasture and I attempted to reduce the size of the portal and splenic veins by the method described for the plication of arteries in this paper. At human autopsies thrombosis of the splenic and portal veins had frequently been observed in cases of splenomegaly. It was our idea that the partial obstruction might lead to a chronic passive congestion of the spleen and perhaps, eventually to splenomegaly. At the end of several months we observed in every instance that the partially occluded veins had resumed their normal size and shape, the points of occlusion being scarcely perceptible. Partial occlusion of the veins when produced by plicating sutures does not persist. In the case of the thick walled arteries the partial occlusion remains. Thus, experimentally as well as clinically, it is observed that veins adapt themselves very much more readily to mechanical disturbances than do arteries.

That partial or complete occlusion of a large artery by compression always leads to the death of the vessel wall seems to be well established. But the rate of vessel destruction due to a ligature or a band depends upon several factors. Our experiments show that a tight ligature or band will cause a more rapid disintegration of the vessel wall

than one loosely applied. One strand of silk or a narrow band will cut through an arterial wall more rapidly than a piece of tape or a wide band applied with the same degree of tension. A tightly applied fine ligature or narrow band produces a greater local anemia of the arterial wall than does an equally tightly applied tape or wide band. It therefore cuts through more quickly, allowing nature a shorter time to repair the damage of the ligature by the formation of fibrous tissue. The plication of an artery is, for the tissues involved, the same as ligation, for the portion of the vessel wall included in the plicating sutures undergoes the same necrosis. Gentle plication over a long segment of the vessel, just as gentle complete occlusion with wide tapes or bands, prolongs the process of dying of the arterial wall until nature's reparative processes make permanent one's operative endeavor.

In dogs we have been able to occlude either partially or completely any artery, except the thoracic aorta, without secondary hemorrhage. Partial occlusion of the thoracic aorta with bands or ligatures cannot be done either in man or dogs without grave danger of fatal hemorrhage. Here the great pressure of the blood and the scant periarterial support hasten death of the arterial wall and prolong the period of repair with fibrous connective tissue. In two experiments a partial occlusion of the thoracic aorta has been accomplished, without hemorrhage, by plicating a long segment of the vessel with mattress sutures of doubled medium silk. In one case the sutures were reinforced with small pieces of muscle.

When balls of fascia are introduced into the lumen of an artery and anchored by a suture of silk, complete occlusion may be accomplished without much damage to the arterial wall save for some atrophy at the site of the plug. This plug produces complete occlusion and at its point of contact with the blood stream it becomes covered with intima. For such a method of occlusion there would seem to be no practical demand, for the arteries that commonly need to be occluded in the human being can be occluded by proper ligation. However, some modification of the method, as for example the introduction of a fascial ball with a small tube through its center, may be the best way of producing a partial occlusion of the human aorta.

The practice of surgeons in the preaseptic era, so beautifully depicted by Ballance and Edmunds, of ligating large arteries over a long distance with loosely tied cords or heavy tapes, has been improved by us only through the elimination of infection. Their practice remains the safest, even though their ideas of the process of destruction and repair were wrong. In Professor Halsted's clinic the complete occlusion of large arteries was always accomplished by the use of two or more loose ligatures of heavy tape or heavy silk, or by wide metallic bands; and secondary hemorrhage never occurred. The use of catgut for the ligation of large arteries was regarded as dangerous and was not permitted. It is highly desirable that the ligature material should remain exactly as placed until the damaged vessel wall becomes completely repaired. In the literature there are several cases of hemorrhage resulting from the ligation of large arteries with strands of catgut. In such cases the giving of the ligatures subjected the arterial wall to high pressure before its damaged wall was completely repaired. To depend on catgut in the occlusion of a large artery involves a risk of secondary hemorrhage that should not be assumed.

Whether the use of plication or fascial plugs will ever become of practical value in the treatment of human conditions remains yet to be seen. That these procedures or some modification of them may help us in our surgery on the human aorta does not seem unlikely.

SUMMARY.

1. Partial occlusion of the aorta of dogs may be produced by Halsted mattress sutures of silk. This occlusion persists.

2. A method of completely occluding the aorta of dogs by means of anchoring fascial plugs in the lumen is described.

3. The vessel wall below and at the site of an occluding fascial plug undergoes marked atrophy.

4. Ligatures used for ligating or plicating large arteries cause death of the arterial wall. The same is true for encircling metallic bands.

5. Catgut is dangerous to use for the ligation of large arteries, since it may give way before the necrosed vessel wall becomes completely substituted by fibrous tissue.

EXPLANATION OF PLATES.

PLATE 11.

FIG. 1. Thoracic aorta of a dog. Lumen reduced to one-third normal size by mattress sutures of silk. *A-B*, innermost line of sutures. An indentation has occurred in the normal wall opposite the sutures. Result after 6 months.

FIG. 2. The black silk suture (*A*) has cut through. In its track there is dense fibrous tissue. A wedge of proliferating intima (*B*) has formed in the crevice. Weigert's elastic tissue stain.

FIG. 3. Photomicrograph of a fascial plug covered by a thin layer of proliferating intima (*A*). At *B*, note the atrophy of the vessel wall. Hematoxylin and eosin stain.

PLATE 12.

FIG. 4. The organization of a thrombus just above a fascial plug. At points *A*, *B*, and *C* where there are breaks in the vessel wall the ingrowth of vascular connective tissue is very striking. Weigert's elastic tissue stain.

FIG. 5. The aortic wall substituted by fibrous tissue. An aluminum band produced the death of the vessel and cut its way into the lumen. Connective tissue growing around the lower edge of the band has substituted for some of the original vessel wall. *A*, site of metallic band. Weigert's elastic tissue stain.

FIG. 6. Longitudinal section through a fascial plug. Weigert's elastic tissue stain. Note the atrophy of the vessel wall at *A*, *B*, and *C*. *D*, a wedge of vascular connective tissue between the vessel wall and ball of fascia. Proximal to the occlusion no atrophy of the vessel wall occurred. In the encircled area the elastic tissue beneath the ligature appears normal, but at this point the nuclei do not stain with hematoxylin and eosin.

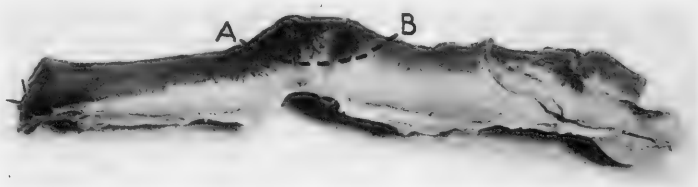

FIG. 1.

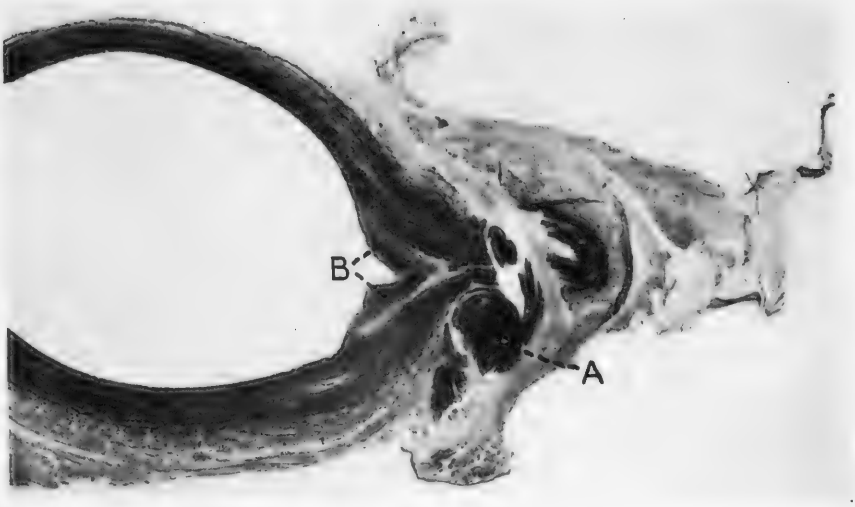

FIG. 2.

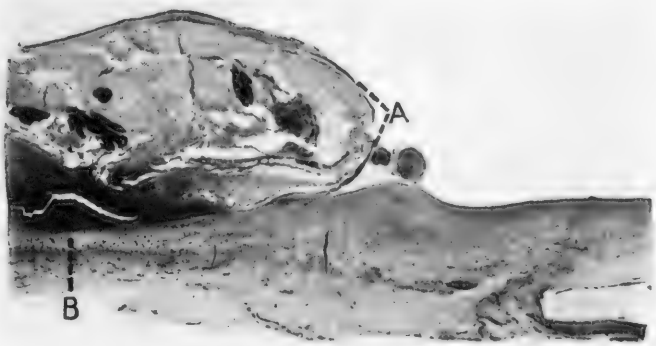

FIG. 3.

(Reid: Partial and complete occlusion of aorta.)

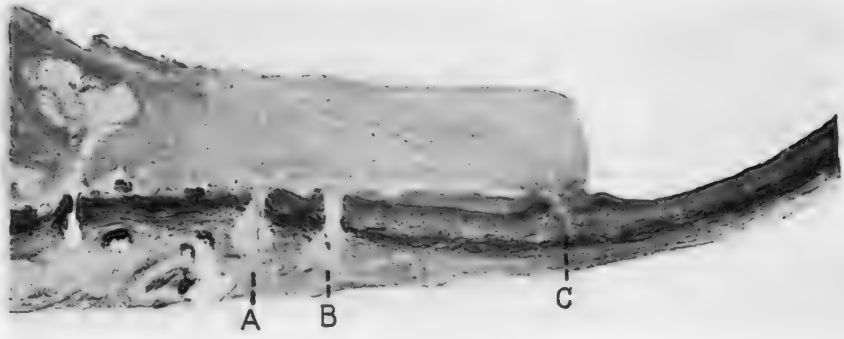

FIG. 4.

FIG. 5.

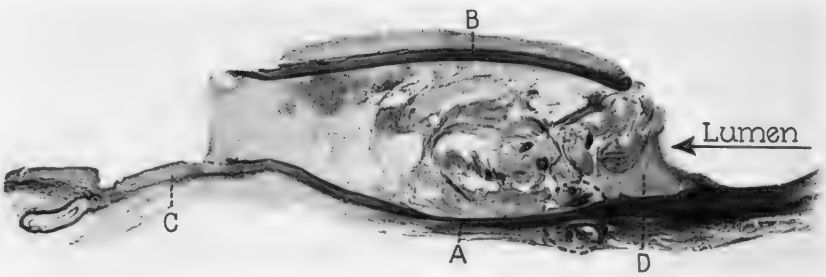

FIG. 6.

(Reid: Partial and complete occlusion of aorta.)

THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

Second Paper.

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The so called "soluble specific substance" of Pneumococcus, it will be recalled, was discovered in 1917 by Dochez and Avery (1) in filtrates from pneumococcus cultures, and was found to be present not only in the intact bacterial cell but also in the body fluids of the infected host. It yielded precipitates only with immune sera of the homologous type of Pneumococcus and, in addition to its high degree of specificity, possessed a stability to heat, enzymes, and many chemical reagents that augured well for its susceptibility to study by the methods of organic chemistry.

In a recent paper by the present writers (2) a preliminary report of such a study was made. A method was described which permitted the concentration of the specific substance of Type II pneumococcus and its separation from a large portion of the accompanying impurities. The resulting product appeared to consist chiefly of a polysaccharide built up of glucose molecules, gave a specific rotation of + 55.7°, contained 1.2 per cent of nitrogen, yielded 79 per cent of reducing sugars on hydrolysis, and still reacted with immune serum when present in as low a concentration as 1:3,000,000. At that time the question was left open as to whether the specific substance itself consisted of polysaccharide or merely accompanied a polysaccharide impurity.

Not only has further evidence on this point now been obtained, but by refinements in the method of purification it has also been possible to reduce the percentage of nitrogen tenfold, and, in a comparative study of the specific substances of Type II and Type III pneumococci, to establish definite chemical differences between them.

EXPERIMENTAL.

Type II Pneumococcus.

1. Method of Purification.

The following modification of the original method of purification of the specific substance is now being used.

8 day cultures of Pneumococcus Type II in meat infusion phosphate broth are concentrated in 21 liter lots, precipitated with alcohol, and put through the initial three layer separation as described in the previous paper. The gummy middle layer is taken up in about 200 cc. of hot water and the resulting mixture from successive lots is kept on ice until 300 to 325 liters of culture have been worked up. The liquid portion of the combined concentrates is decanted from any salts that have separated and these are dissolved in hot water. The solutions are mixed and run through a Sharples supercentrifuge, washing through thoroughly with water. In a representative run, the volume of the resulting somewhat turbid solution was 4,200 cc. and complete precipitation of the active material required 5.5 liters of alcohol. The mixture is allowed to stand overnight, the clear supernatant liquid is siphoned off, and the precipitate again centrifuged at high speed. The top and bottom layers are discarded and the middle layer is taken up in hot water. The turbid solution is centrifuged and the precipitate washed twice with water and then discarded. 20 gm. of sodium acetate are then dissolved in the solution and washings, since if an insufficient concentration of electrolyte is present an unduly large amount of alcohol must be used to precipitate the active material. The aqueous solution, which should now measure about 1.5 liters, requires 2.5 to 3 liters of alcohol for precipitation. The upper layer is again drawn off and the precipitate treated as before, any bottom layer formed after centrifugation being discarded. The active material is dissolved in water, centrifuged, and the solution, at a volume of 1 liter, treated with 1.5 gm. of sodium acetate and precipitated with 1.5 liters of alcohol. The precipitate is treated as before, after which the aqueous solution of the specific substance, at a volume of about 500 cc., is acidified with acetic acid until no further permanent precipitate forms. The mixture is then repeatedly centrifuged until most of the precipitate separates as a solid cake, from which the almost clear supernatant liquid can be poured off. The precipitate is washed with water and discarded and the solution and washings, at a volume of 750 cc., are treated with 10 gm. of sodium acetate and precipitated with 1 liter of alcohol. The precipitate is centrifuged off, redissolved in hot water containing a little acetic acid and sodium acetate, centrifuged if necessary, and, at a volume of 600 cc., precipitated with 1,100 cc. of alcohol. After centrifugation the active substance is dissolved in water, the solution is treated with 50 cc. of 10 per cent aqueous sodium hydroxide, centrifuged if necessary, and made up to 500 cc.

Alcohol is then cautiously added with vigorous stirring until a slight permanent turbidity remains. This is centrifuged off and the clear solution poured from the slight precipitate which generally consists of inactive material. Addition of alcohol is then continued until all of the specific substance has been precipitated, about 700 cc. in all being required. The solid is centrifuged off, dissolved in cold water, centrifuged again, and to the supernatant and washings are added 7 gm. of sodium acetate and 25 cc. of normal sodium hydroxide. The volume is adjusted to 400 cc. and the specific substance precipitated with 600 cc. of alcohol. After centrifugation it is redissolved in water and the solution acidified with acetic acid. At a volume of 180 cc. the solution gives a slight precipitate with about 90 gm. of ammonium sulfate, consisting mainly of impurities precipitable by tannic acid. After centrifugation the supernatant is saturated with ammonium sulfate, precipitating most of the active material as a gum which can be separated by centrifugation. The precipitation with ammonium sulfate is repeated twice, each time from a smaller volume. Small amounts of active material remain in solution and may be recovered by dialysis, concentration to very small volume, and saturation with ammonium sulfate. The main precipitate is dissolved in water and dialyzed in parchment bags against running water until most of the sulfate is removed. The solution is then concentrated to about 100 cc., cooled, diluted with 25 cc. of 1:1 hydrochloric acid, and again subjected to dialysis. The bags are finally transferred to several changes of distilled water until they are entirely free from chloride and sulfate. The solution is then concentrated to dryness on the water bath and the residue taken up in about 100 cc. of hot water. After centrifugation the solution is poured into 1,200 cc. of redistilled acetone, precipitating the specific substance as a white, asbestos-like mass which crumbles readily when dried. The yield is about 4.5 gm. from 300 to 325 liters of culture. From the acetone solution only a very little dark-colored residue is obtained on evaporation.

As stated in the previous paper, variations in the exact volumes given are often necessary with different lots of cultures, but this will occasion little difficulty if all fractionations are controlled by the specific precipitin test.

The properties of the specifically reacting polysaccharide material obtained in this way are summarized in Table I, in which all figures except those in the last two columns are calculated on the ash-free basis. Preparations 21 A and 24 were prepared by the above method,[1] while in No. 21 the alkaline purifications were omitted.

It thus appears that the specific soluble substance of Type II pneumo-

[1] Recent tests have indicated that the calcium oxide found as ash in the various preparations may have been derived at least in part from the parchment bags and experiments are now in progress in which collodion membranes were substituted.

coccus, in its present state of purity, consists of polysaccharide material containing 0.2 per cent or less of nitrogen, yielding about 75 per cent of reducing sugars on hydrolysis, and rotating the plane of polarized light about 55° to the right. It contains about 47 per cent of carbon

TABLE I.

Preparation No.	Specific rotation.	N	Reducing sugars on hydrolysis.*	C	H	Ash.	Precipitation with anti-pneumococcus serum.†
Type II pneumococcus.							
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	
21	+55.2°	0.46	70.5	46.8	6.0	3.7	1:10,000,000
21 A	+55.8°	0.20	67.2			3.2	1:5,000,000
24	+58.2°	0.16	74.8			3.7	1:2,000,000
22 A	+52.2°	0.41	65.8			4.2	1:5,000,000
22 B	+58.5°	0.31	62.6			4.2	1:5,000,000
22 F	+53.9°	0.12	72.5			3.5	1:2,000,000
22 H	+56.3°	0.05	76.1			4.1	1:2,000,000
23	+53.9°	0.39	62.3			6.5	1:5,000,000
24	+58.2°	0.16	74.8			3.7	1:2,000,000
24 A	+50.0°	1.0	74.3			4.0	1:2,000,000
24 C	+50.0°	0.19	75.0			4.0	1:5,000,000
Type III pneumococcus.							
27	−33.0°	0.11	73.0	42.3	5.2	0	1:2,000,000
28	−34.0°	0.05	73.0	42.6	5.6	0	1:3,000,000
31	−34.1°	0.10	74.0			0.2	1:3,000,000

*Calculated as glucose.

†After 2 hours at 37° and overnight at 4°.

and 6 per cent of hydrogen, and is free from sulfur and phosphorus, as determined on 0.2 gm. portions. It contains about 4 per cent of ash, left on ignition as calcium oxide, indicating perhaps that the product isolated is the salt of an acid. It gives no color with iodine and in 0.5 per cent solution is not precipitated by heavy metal salts

except basic lead acetate and, strangely enough, uranyl nitrate. It gives no biuret reaction, nor does it precipitate with phosphotungstic acid or tannic acid.

It will be recalled that in the first paper glucosazone was isolated from the products of hydrolysis of the specific substance. Since this could have been derived from glucose, fructose, or mannose, a clue was sought in the determination of the optical rotation during the course of the hydrolysis. In one experiment in which the initial specific rotation was + 62° in one-half normal hydrochloric acid, the identical value was obtained after $1\frac{1}{2}$ hours boiling. Since the equilibrium value of the specific rotation of glucose is +52.8°, while the values for mannose and fructose are much lower, it would appear that the polysaccharide contained in the specific soluble substance of Type II pneumococcus is built up of glucose units.

2. Attempts to Separate the Specific Substance from the Carbohydrate.

Efforts at dissociation of the specific substance from the polysaccharide portion of the material were directed along the following lines: (*a*) hydrolysis of the carbohydrate; (*b*) precipitation of the specific substance with immune serum; (*c*) precipitation with uranyl nitrate; (*d*) precipitation with basic lead acetate; and (*e*) precipitation with safranine.

(a) Relation of Specificity to Acid Hydrolysis.

The followng experiment shows, in a rough, qualitative way, how the specific reaction diminishes only as the polysaccharide is hydrolyzed by strong acid, and also illustrates the remarkable stability of the specific substance to strong acid in the cold.

1:1 Hydrochloric Acid Used at Room Temperature.

Original concentration of Preparation 21, 1:1,000.

Test No.	0	1	2	3	4	5
Time	0	2 hrs.	19 hrs.	2 days.	3 days.	6 days.
Immune serum	+++	+++	+++	++±	+	−
Cu reduction	−	−	−	+	++	+++

(*b*) *Precipitation of the Specific Substance with Immune Serum.*

0.25 gm. of Preparation 24 was dissolved in 250 cc. of 0.9 per cent salt solution and treated with Type II immune horse serum until no further precipitate formed. 900 cc. of serum were required. After several hours at 37° the mixture was centrifuged and the gelatinous precipitate ground up in a mortar as well as possible with saline, diluted to 200 cc., and centrifuged. Washing with saline was repeated, both washings failing to react with immune serum. The precipitate was ground up with water and the suspension diluted to about 600 cc., acidified slightly with acetic acid, and boiled for 10 minutes. The resulting coagulum retained all of the specific substance, however, so that some other scheme of dissociation became necessary. After considerable experimentation it was found that the specific substance could be leached out by repeated extraction on the water bath with approximately normal ammonium hydroxide. The opalescent solutions so obtained, which contained much protein material, were neutralized with acetic acid, combined, concentrated on the water bath, centrifuged from precipitated protein derivatives, and dialyzed. The resulting solution was concentrated to 25 cc., treated with 2 gm. of sodium acetate, and precipitated with alcohol, the process being repeated twice from slightly smaller volumes. The precipitated specific substance was then dissolved in water, centrifuged, and evaporated dry on the water bath. The residue was taken up in water, centrifuged, and the process repeated until only a trace of insoluble material remained after drying. The residue was then dissolved in 4 to 5 cc. of water and poured into 60 cc. of redistilled acetone. The specific substance precipitated as a white, fibrous mass, and was washed with acetone and dried. The yield was 0.1 gm.

A comparison of the recovered specific substance (No. 24 A) with the original (No. 24) is given in Table I. It will be seen that precipitation of purified soluble specific substance with the homologous immune serum, followed by recovery of the specific substance from the immune precipitate, leads to a product essentially the same in optical activity, reactivity with immune serum, and percentage of reducing sugars on hydrolysis; in other words, a polysaccharide derivative greatly resembling the starting material. That the higher percentage of nitrogen in the recovered product is due to contaminating protein degradation products originating in the relatively enormous amounts of protein from which a small amount of specific material had to be separated, is indicated by the fact that the recovered specific substance, in contradistinction to all other preparations, gave a weak biuret test and a slight haze with tannic acid.

(*c*) *Precipitation with Uranyl Nitrate.*

To a solution of 2.2 gm. of a mixture of equal parts of Preparations 22 A and 22 B in 440 cc. of water, 5 per cent uranyl nitrate solution was cautiously added, with vigorous stirring, until present in slight excess. The mixture, from which a heavy, curdy yellow precipitate had settled, was neutralized to litmus with sodium hydroxide and allowed to stand overnight in the ice box. The supernatant liquid, after removal of the excess of uranyl ion with ammonia, reacted only feebly with Type II serum, but was concentrated to small bulk, treated with 1:1 hydrochloric acid in excess, and dialyzed against running water, and finally concentrated to dryness. 0.018 gm. of residue was obtained. The material was partially soluble in water, feebly dextrorotatory, reduced Fehling's solution after hydrolysis, and, in 1:200 solution, reacted strongly with Type II serum. It apparently consisted largely of specific substance that had escaped precipitation, together with accumulated impurities.

The specific soluble substance was liberated from the main uranium precipitate by repeated extraction on the water bath with 200 cc. of normal ammonium hydroxide solution. The centrifuged solutions were neutralized, combined, made slightly alkaline with ammonia, and heated to boiling, after which the small amount of ammonium uranate which separated was centrifuged off. The supernatant liquid was concentrated *in vacuo* to about 800 cc. and heated on the water bath for 1 hour after addition of 10 gm. of sodium acetate, 5 cc. of acetic acid, and a concentrated solution of 3 gm. of sodium dihydrogen phosphate. Small amounts of uranium still in solution were precipitated as uranyl phosphate, which was filtered off after the mixture had been cooled. The filtrate was neutralized to litmus, concentrated to 400 cc., filtered from a slight precipitate, cooled, and made strongly alkaline with 10 per cent aqueous sodium hydroxide. The resulting precipitate was centrifuged off in the cold and the clear supernatant neutralized with hydrochloric acid, concentrated to 150 cc., and dialyzed against running water with occasional additions of 10 cc. of 1:1 hydrochloric acid during the few days of dialysis. After removal of all phosphate and chloride ions the solution was concentrated to 20 cc., centrifuged from a slight precipitate, and poured into 210 cc. of redistilled acetone. The precipitate was washed with acetone and dried. The yield was 1.4 gm. and the color faintly yellow.

A comparison of the properties of the product (No. 22 F) with the original material (Nos. 22 A and B) shows that again a polysaccharide of substantially the same properties and specific activity was recovered.

(*d*) *Precipitation with Basic Lead Acetate.*

1.93 gm. of Preparation 24 were dissolved in 500 cc. of water and precipitated with a slight excess of basic lead acetate solution. The mixture was allowed to stand in the ice box overnight and was then centrifuged. The supernatant, freed from lead with hydrogen sulfide, and dialyzed, left only 0.7 mg. on evaporation, showing that the original material had been quantitatively thrown down.

The main precipitate was freed from lead by precipitating the metal ion as chromate, a preliminary experiment having shown difficulties in the customary removal of lead as sulfide owing to the tendency of the specific material to act as a protective colloid. The basic lead acetate precipitate was accordingly dissolved in dilute acetic acid and the very viscous solution diluted to 500 cc. with water. After addition of 10 gm. of sodium acetate the solution was heated on the water bath and treated with an excess of half normal potassium dichromate solution. After 5 minutes heating it was allowed to cool and was repeatedly centrifuged at high speed until the finely divided lead chromate had settled to the bottom. The yellow supernatant was dialyzed in parchment until it no longer gave a test for chromate ion with barium chloride, although the solution remained faintly yellow. During concentration on the water bath the color changed to a pale green, indicating the presence of traces of chromium ion, and part of the specific substance separated as a scum which had to be redissolved separately in hot water. After an additional dialysis in the presence of strong hydrochloric acid, the solution was further concentrated *in vacuo* to about 65 cc. and poured into 750 cc. of redistilled acetone. 0.82 gm. of solid separated in a somewhat more dense form than in the case of the other preparations.

The product (No. 24 C), as will be seen from Table I, was again essentially the same type of polysaccharide derivative as the original material and the preceding preparations.

(*e*) *Precipitation with Safranine.*

In recent work by Marston (3) safranine is used as a precipitating agent for trypsin and pepsin, resulting in a considerable purification of the enzymes. While safranine is far from a specific precipitant for enzymes, throwing down, as it does, such widely diversified types of substances as serum protein and gum arabic, it nevertheless seemed of interest to study its effect on the soluble specific substance, since as an azine dye, it represented a totally different type of precipitant from the immune serum or metallic salts previously used. A preliminary experiment showed that in suitable concentration the specific substance was quantitatively precipitated by safranine.

0.3 gm. of Preparation 22 F (Table I) was dissolved in 10 cc. of water and treated with a 1 per cent aqueous solution of Grübler's safranine until no further precipitate formed. The dark red, gummy precipitate was washed with 1 per cent safranine solution, dissolved in one-half normal hydrochloric acid, and shaken repeatedly with butyl alcohol, with occasional additions of water to make up for the amount taken up by the solvent. In order to remove all but traces of the dye it was necessary to make the solution alkaline and shake with butyl alcohol, and finally to repeat the process on the acid side. The aqueous solution was then neutralized to litmus, concentrated to dryness on the water bath, taken up in water, centrifuged from traces of insoluble material, and dialyzed after addition of 1:1 hydrochloric acid. The chloride-free solution was concentrated to dryness and the residue taken up in 5 cc. of water. The solution was centrifuged and poured into 60 cc. of acetone, precipitating 80 mg. of faintly pink, fluffy material.

As will be seen from No. 22 H, Table I, precipitation by safranine failed to alter the character of the specific polysaccharide material.

(*f*) *Preparation from Intact Pneumococci and Purification by Adsorption.*

It was thought that if a dissociation of polysaccharide and specific substance were possible, especially if the carbohydrate were derived from the broth culture medium, it might take place more readily if both the source of the crude specific substance and the method of purification were altered. Accordingly the intact bacterial cell was chosen as the source of the specific substance, and this was removed from solution by adsorption on aluminium hydroxide, a method used with success by Willstätter (4) in the purification of invertase.

120 liters of an 18 hour broth culture of Type II pneumococci were treated in ten lots as follows:

The pneumococci were centrifuged off at high speed, taken up in about 1 liter of 0.85 per cent NaCl solution, and treated with the minimum amount of bile necessary to effect complete solution of the bacterial cells. This solution was acidified with dilute acetic acid to precipitate the bacterial protein, and after removal of this by centrifugation, was neutralized to litmus and autoclaved. Each lot of filtrate, about 1.5 liters in volume, was shaken overnight with an especially bulky form of aluminium hydroxide, about 40 gm. being necessary to remove the specific substance from solution in each case. The hydroxide was allowed to settle, and, after the supernatant liquid had been siphoned off, was washed once with 1.5 liters of water. This was also siphoned

off, and the precipitate extracted overnight in the shaking machine with 1 liter portions of 2 per cent disodium phosphate solution. Three extractions were usually sufficient to remove the specific substance from the aluminium hydroxide. The phosphate extracts were neutralized to litmus with acetic acid, concentrated to small bulk on the water bath, and combined as the different lots were worked up. The total concentrate was then warmed to dissolve salts which had crystallized and any aluminium hydroxide present was filtered off. The filtrate was then precipitated with alcohol and the precipitated specific substance purified by repeated fractionation as in the original method. The resulting opalescent aqueous solution was finally dialyzed until free from salts and passed through a Berkefeld W filter, and concentrated to dryness on the water bath. The residue was taken up in water, centrifuged, and the solution concentrated to 10 to 15 cc. and poured into 160 cc. of acetone. 0.32 gm. of a voluminous, white, fluffy precipitate was obtained.

Although the broth culture medium as a source of the specific substance was eliminated, and the method of purification radically altered, the properties of the specific substance recovered (No. 23, Table I) remained entirely analogous to those of the other preparations discussed in this section, in each of which highly purified material was taken as a starting point and was treated in a different way.

Efforts at splitting the specific substance-polysaccharide complex, if such it be, with the aid of enzymes have been made, but have so far proved unsuccessful, as the polysaccharide is not attacked by the ordinary carbohydrate-cleaving enzymes. Several molds have also been used without success.

Type III Pneumococcus.

Little work was necessary on the specific soluble substance of Type III pneumococcus to show that marked chemical differences existed between it and the corresponding substance of Type II. 8 day broth cultures were used in this case also and the method of concentration and purification was the same up to the last steps, differences occurring only in the tenacity, appearance, and greater quantity of the precipitates of active material.

It then developed that not only is the Type III specific substance precipitated by heavy metal salts such as those of silver, mercury, and copper, which do not precipitate the Type II substance, but that, in conformity with this, the Type III substance is the soluble alkali

or alkaline earth salt of an *insoluble* strong acid which is thrown out of solution in the presence of an excess of strong hydrochloric acid.

Preparations 27 and 28 (Table I) were obtained from material which had been carried through the usual method of fractionation, including the final dialysis. In No. 27 the specific substance was first precipitated by copper chloride and freed from copper by treatment with hydrogen sulfide, while in No. 28 this step, later shown to be unnecessary, was omitted. The free specific acid itself was obtained as follows, the quantities given referring to the material from 300 to 325 liters of broth:

The final, dialyzed solution of the soluble substance, at a volume of about 600 cc., is treated with 100 cc. of 1:1 hydrochloric acid. After a preliminary interval the specific acid begins to separate slowly in fibrous masses which form gelatinous clumps and do not appear to be crystalline under the microscope. Frequent stirring is necessary to prevent the formation of a stiff jelly. The mixture is allowed to stand overnight in the ice box and is then filtered on a 7 inch Buchner funnel, pressed out with a flat top glass stopper, and washed twice with normal hydrochloric acid. From the filtrate in the case of Preparation 28 another polysaccharide was isolated after dialysis. It had a specific rotation of +185°, gave 100 per cent of reducing sugars on hydrolysis, was readily fermented by ptyalin, contained 0.05 per cent of nitrogen, and gave a purplish red color with iodine. It was therefore indistinguishable from erythrodextrin or glycogen, and was probably derived from the broth culture medium.

After washing on the funnel with normal hydrochloric acid the specific acid is washed repeatedly with 50 per cent alcohol, the suction being disconnected and the substance allowed to soak up the alcohol at each washing. When the washings no longer contain chlorine ion, the specific acid is washed several times with redistilled acetone and dried *in vacuo*, being warmed from time to time to accelerate the progress. The yield varies from 6.5 to 9 gm., and the substance is ash-free.

Since the properties of the specific acid were so characteristic and even permitted removal of an accompanying carbohydrate, it was thought that the long scheme of purification might be shortened with its aid. Accordingly, Preparation 31 was worked up in this way:

After the third reprecipitation with alcohol and subsequent removal of additional impurities by precipitation with acetic acid the centrifuged solution, at a volume of 800 cc., was treated with about 200 cc. of 1:1 hydrochloric acid, occasionally stirred, and allowed to stand overnight in the ice box. The precipitate was filtered off, washed with a little normal hydrochloric acid, suspended in water, and dissolved with the aid of a slight excess of sodium hydroxide. The resulting somewhat turbid solution was acidified faintly with hydrochloric acid,

diluted to about 800 cc., and run through an 8 inch Berkefeld N candle, washing through with much water. The resulting clear solution was concentrated to about 600 cc. and reprecipitated as in the case of Preparation 28. The yield of slightly yellowish material was 9.6 gm. The precipitation of the Type III substance by hydrochloric acid is not entirely quantitative, but the loss is small in proportion to the degree of purification attained.

It will be seen from the remarkable uniformity of the figures in Table I for Preparations 27, 28, and 31 that one is apparently dealing here with a much more definite chemical entity than in the case of the Type II substance, in which, in the absence of so characteristic a property which can be made use of in the process of purification, the variation in the findings is of a much wider range. While the Type II specific substance rotates the plane of polarized light about 55° to the right, the insoluble Type III acid, dissolved by neutralization with sodium hydroxide, gives a rotation of *minus* 34°. Its activity with homologous antipneumococcus serum and the percentage of reducing sugars found on hydrolysis are about the same as in the case of the Type II substance, while the nitrogen content is so low as to be practically negligible. The carbon content is about 4 per cent less and the hydrogen content about 0.5 per cent less. 0.1 gm. of the acid requires about 0.3 cc. of normal sodium hydroxide for neutralization to litmus. The Type III substance, as well as the Type II, gives no color with iodine, no biuret reaction, and no precipitate with tannic acid, but it is precipitated not only by uranyl nitrate and basic lead acetate, but also by many other heavy metal salts which do not affect solutions of the Type II substance. There is thus no question of the marked differences in chemical properties of the two substances, and some insight into the cause of these differences is afforded by the behavior of the Type III substance on hydrolysis.

2.0 gm. of Preparation 28 (corresponding to 1.92 gm. of anhydrous substance) were suspended in water and dissolved by the gradual addition of normal sodium hydroxide, 6.5 cc. giving a solution neutral to litmus when all the acid had dissolved. The solution was made up to 192 cc. and showed a specific rotation of −36.5°. After mixing with 192 cc. of normal hydrochloric acid the rotation was unchanged but on boiling it gradually decreased to 0° and became positive, tending to become constant at about +23° after 5 hours. The hydrolyzed solution was cooled, neutralized to Congo red with 10 per cent sodium hydroxide, concentrated *in vacuo* to 164 cc., and divided into two portions.

By the action of phenylhydrazine on the first portion an osazone was obtained, crystallizing in balls of microscopic needles. After washing with methyl alcohol the yield was 0.062 gm. The osazone softened above 180° and decomposed above 195°, and was levorotatory, but was so insoluble in the usual alcohol-pyridine mixture that a quantitative determination of the rotation was impossible. The remaining half of the hydrolysis mixture was almost neutralized with sodium hydroxide, treated with 1 to 2 gm. of sodium acetate, and to the solution 1.5 gm. of freshly recrystallized *p*-bromophenylhydrazine hydrochloride were added. The solution was then heated on the water bath and filtered from the small amount of reddish tar which separated almost at once. A crystalline osazone soon separated. After 40 minutes heating, the mixture was allowed to cool, and the partially crystalline osazone was filtered off, washed with water, and ground up with glacial acetic acid, finally placed in the water bath for a few moments. The mixture was quickly cooled, diluted with several volumes of ether, and allowed to stand overnight in the ice box. A gelatinous precipitate over the heavy crystalline sediment was poured off and the crystals were filtered off and washed first with glacial acetic acid and finally with ether. The yield was 0.040 gm. When rapidly heated, the *p*-bromophenylosazone derivative softened and darkened at about 180° and melted with decomposition at about 190°. It gave a clear, pale yellow solution in pyridine-alcohol (3:2) with an initial specific rotation of 280° and a final value of $[\alpha]_D$ −230°. According to Neuberg (5) glucuronic acid forms a derivative with *p*-bromophenylhydrazine characterized by an extraordinarily large levorotation, but his product had different properties from the one obtained above. However, a portion of the hydrolysis liquid gave a strong orcinol test for glucuronic acid.[2]

DISCUSSION.

As regards the soluble specific substance of Pneumococcus Type II, it has proved possible to start with purified carbohydrate material, containing 0.2 per cent or less of nitrogen and still capable at a dilution of one part in 2 to 5 million of precipitating immune serum, and with such a product by a number of diverse procedures, to recover in each instance a substance almost identical with the starting material in optical rotation, percentage of nitrogen, percentage of reducing sugars on hydrolysis, and specific activity. Whether the specific substance is precipitated by immune serum, by uranyl nitrate, by basic lead acetate, or by safranine, precipitants of three totally

[2] The writers wish to express their heartiest thanks to Dr. P. A. Levene for his generously given time and assistance in connection with this and other phases of the work, and to Miss I. Weber and Dr. W. A. Jacobs for their help, as well.

unrelated types; whether the intact bacterial cell or the broth culture medium is used as the source of the material; or whether the method of purification is based on simple fractional precipitation, or on adsorption, the product recovered is always essentially the same, and always largely a polysaccharide. Coupled with this are the facts that the specificity of this extraordinarily stable material does not diminish on treatment with strong acid in the cold until reducing sugars begin to appear, and that by the same technique it is possible from Type II and Type III pneumococci to isolate *different* polysaccharide derivatives of equal specific activity. If the polysaccharide encountered were an impurity derived from the broth culture medium, contaminating an unknown specific substance, it might be expected to be the same, or at least very similar, in both instances.

It is thus becoming increasingly difficult to believe that the soluble specific substance is not actually the polysaccharide derivative itself, for there is now accumulated a considerable mass of evidence in favor of this view.

The study of the chemical nature and biologic specificity of this polysaccharide material furnishes a basis for the better understanding of the immunological relationships of the bacterial cell. The serological specificity of the various types of Pneumococcus is intimately related to, if not solely dependent upon, the presence of this specific cell constituent. The synthesis of this polysaccharide material is a cellular function highly developed in those strains of pneumococci which are most capable of multiplying in animal tissues. This substance apparently bears a significant relationship not only to type specificity but to virulence and capsular development. The elaboration of this soluble specific substance during growth in the animal body is so marked that its presence may be detected in the body fluids of experimentally infected rabbits and in the blood and urine during the course of the spontaneous disease in man.

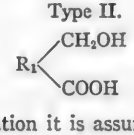
As pointed out in a preceding paper (6), the pneumococcus cell contains among other constituents two substances which are separable and distinct chemically and which possess different properties immunologically. One of these cell constituents is protein in character. Further studies on the biological specificity of pneumococcus protein are now in progress. It need only be mentioned here that

serologically and antigenically this protein fraction seems to be less specific as to type than is the intact bacterial cell. The second, or carbohydrate fraction, the chemical nature of which is dealt with in this communication, is highly and specifically reactive only with antibacterial serum of the same type of Pneumococcus as that from which the substance is derived. Although this bacterial polysaccharide is specifically reactive with antibody produced in response to immunization with the intact organism, it is itself, when dissociated from combination with other cell constituents, but slightly if at all capable of inducing antibody formation. The biologic differences in the specificity of the soluble specific substances of Type II and Type III pneumococcus are reflected in differences in their chemical constitution.

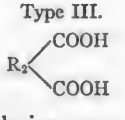
The chemical difference between the soluble specific substances of Pneumococcus Types II and III are so marked as to cause astonishment that analogous fractions of closely related microorganisms can have such widely divergent properties. While both consist of about 75 per cent polysaccharide[3] and 25 per cent of unknown constituents, the Type II substance is dextrorotatory, the Type III substance levorotatory; the former, if the salt of an acid, is the salt of a soluble acid, while the latter is the salt of an extremely strong, difficultly soluble acid, so strong, indeed, that in very dilute aqueous solution it turns Congo red paper blue. The polysaccharide portion of the Type II specific substance appears to be built up of glucose units, while that of the Type III substance seems, on the basis of preliminary evidence, to be composed either of glucuronic acid itself or of some analogous acid. The lower percentages of carbon and hydrogen in the Type III substance are also in harmony with this view, since the replacement of $-CH_2OH$ groups by $-COOH$ (the difference between glucose and glucuronic acid) would have this effect.

The differences between the two specific substances, if they may be assumed to be chemical individuals, may be presented by the following two expressions:

Type II.



Type III.



[3] In the calculation it is assumed that the reducing power of the sugars obtained on hydrolysis is the same as that of glucose.

It is hoped soon to report on the positive identification of the sugar unit of the Type III substance, on the nature of the unknown portion of the specific substance, and on the soluble specific substance of Type I pneumococcus.

SUMMARY.

1. The method for the concentration and purification of the soluble specific substance of Pneumococcus has been improved.

2. Highly purified specific substance of Type II pneumococcus of polysaccharide nature is shown to be recovered essentially unchanged after precipitation by immune serum, by uranyl nitrate, by basic lead acetate, or by safranine.

3. Marked chemical differences are shown to exist between the specific substances of Type II and Type III pneumococcus, although both react as polysaccharides.

4. The weight of evidence is considered to be in favor of the view that the specific substances of Pneumococcus Types II and III are actually polysaccharide derivatives.

5. The immunological significance of the foregoing view is discussed.

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STUDIES ON THE TWORT-D'HERELLE PHENOMENON.

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Since the first publications of Twort (1) and d'Hérelle (2), and the important contributions of Bordet and Ciuca (3) and of Kabeshima (4), experimental studies of the Twort-d'Hérelle phenomenon have been numerous, but there is still no generally accepted interpretation of it. However, as time passes the hypothesis of d'Hérelle of the existence of an ultra parasite seems to find less favor. The bibliography of the subject is already quite extensive. For a complete review we need only refer to the monograph of d'Hérelle, the critical articles of Maitland (5) and Davison (6), and the works of Seiffert (7) and Kuttner (8).

The present studies may be divided into two series. In the first of these we have repeated some observations on the phenomenon in general and have given special attention to certain peculiarities of the lytic principle which have aroused our interest. In the second series the antigenic properties of the filtrates of lysed cultures[1] have been studied.

Origin and Characteristics of the Lytic Principle Studied.

We have employed in these experiments a lytic principle isolated from the feces of a normal rabbit. We have not made a systematic study on the frequency of bacteriophage in the intestinal tract of normal rabbits, but frequent examinations indicate that bacteriophage is constantly present in the feces of caged laboratory rabbits. To

[1] During the preparation of this report there has appeared a paper on the same subject by Weiss and Lloyd (Weiss, E., and Lloyd, A., *J. Infect. Dis.*, 1924, xxxiv, 317). Their results are in partial accord with ours.

obtain the lytic principle we have employed the technique described by d'Hérelle.[2]

Some particles of fecal matter are ground up with a little bouillon in a sterile mortar. This coarse suspension is added to 70 or 80 cc. of sterile bouillon in a small Erlenmeyer flask and allowed to incubate at 37°C. overnight. Next day it is filtered first through paper and then through a Mandler filter. It is then distributed into sterile tubes and each tube is inoculated with a culture of the bacteria to be tested. After incubation for 24 hours the contents of each tube are again filtered separately. A small quantity (0.5 to 1.0 cc.) of this second filtrate is added to the young bouillon culture of the same organism, and incubated at 37°C. After 2 hours 2 or 3 drops of this mixture are flowed over the surface of a sterile agar slant. After incubation overnight the extent of lysis is indicated by the number of clear spaces ("plages" of d'Hérelle) which appear in the bacterial growth on the slant.

The following strains of bacteria were employed; *B. dysenteriæ* Shiga (two strains), *B. dysenteriæ* Flexner (two strains), *B. typhosus*, *B. paratyphosus* A and B, *B. choleræ suis*, *B. coli communis*, *B. coli communior*, and *B. lactis aerogenes*, all stock strains found in this laboratory. Strain Shiga 73 was isolated in 1919 from a fatal case of dysentery; it has been maintained on artificial media and has never shown spontaneous lysis. The typhoid strain (Rawlings) was one used in many laboratories of the United States for the preparation of antityphoid vaccine.

At the beginning of these studies we obtained lytic filtrates for one or many of the bacteria tested. The most active filtrate was selected for further experiment. The first series of titrations gave the following results. The lytic principle was active in a dilution of 1:1,000,000 for Strains Shiga 73 and Flexner 57, 1:10,000 for the other Flexner strain, and 1:1,000 for the typhoid. It was inactive for the other strains. I started immediately to increase the activity of the active principle by successive inoculation and filtration of cultures. For these experiments the attempt was limited to two strains, Shiga 73, which was the most sensitive to lysis, and the Rawlings typhoid strain, which was the least sensitive. After twenty successive passages a titration of the ultimate filtrates gave the following results. The Shiga filtrate was active for its homologous strain in a dilution of about 1:10,000,000,000 but no longer possessed any lytic activity for the typhoid strain. The typhoid filtrate was similarly active in a dilution of 1:10,000,000,000 for the typhoid strain but was only feebly

[2] d'Hérelle (2), p. 24.

active for the Shiga strain (in a dilution of about 1:1,000). With these two filtrates animals were immunized and the experiments to be described were carried out.

Filtration Experiments with Collodion Membranes.

Maisin (9) and Wollman (10) have studied the filterability of bacteriophage through collodion membranes. We have repeated the experiments, following about the same technique.

A collodion sac is mounted on a glass tube of about 6 mm. diameter. It is filled with bouillon and half immersed in a larger test-tube containing some of the same bouillon. The small tube attached to the collodion sac was allowed to pass up through the cotton plug of the test-tube and to project 4 or 5 cm. The projecting end of the small tube was also plugged with cotton. The assembled apparatus was sterilized in the autoclave at 120°C. After sterilization the contents of the collodion sac were removed by means of a bulb pipette and replaced by active filtrate. The bouillon outside the collodion sac was inoculated with the sensitive organism. After incubation for 6 hours a few drops of the culture in the outer tube were inoculated onto the surface of an agar slant. This process was repeated after 18 hours and at the same time an agar slant was inoculated with some of the contents of the collodion sac to test its sterility. If the bacteriophage had passed through the membrane the cultures made with material from the outer tube should have shown the characteristic plages on the agar slants, if not, the culture was regarded as normal. If the contents of the collodion sac were found not to be sterile the experiment was disregarded, as it was considered that the membrane had perforated.

This experiment was carried out many times and with membranes of different thickness. The passage of the lytic principle through collodion membranes was never observed. Experiments with sacs made with collodion of less than 4.5 per cent were not satisfactory because these sacs were not resistant to autoclaving or handling. Only sacs made with collodion solutions of 4.5 to 5.0 per cent were able to withstand these procedures.[3]

Attempts to Inactivate the Active Principle by Suspensions of Cholesterol, Lecithin, or Killed Bacteria.

Otto, Munter, and Winkler (11, 12) and Seiffert (7) have published studies relative to the ability of certain suspensions to absorb the

[3] The collodion used in these experiments was known as Parlodion, obtained from Arthur H. Thomas Company of Philadelphia.

lytic principle. For this purpose they have employed animal charcoal, bolus alba, and Kieselgur. We have made comparative experiments with suspensions of cholesterol, lecithin, and suspensions of typhoid and dysentery bacilli killed by heat.

The suspensions of cholesterol and lecithin in distilled water were prepared from acetone (for cholesterol) or alcohol (for lecithin) solutions. The acetone or alcoholic solutions were titrated, and after the addition of distilled water the acetone or alcohol was evaporated off on the water bath. The aqueous suspensions contained 0.25 mg. of cholesterol or 1.0 mg. of lecithin per cc. The bacterial suspensions were made quite thick, being composed of the growth from ten agar slants washed off in physiological salt solution, centrifuged, and resuspended in 5 cc. of salt solution. 0.1 cc. of the lytic filtrate was added to 5 cc. of the suspension of cholesterol, lecithin, or killed bacteria. The mixtures were incubated at 37°C. for 3 hours and then refrigerated 24 or 48 hours, after which they were centrifuged and the lytic action of the supernatant fluid titrated by dilution in the usual manner. No appreciable diminution of the activity of the filtrates was observed after exposure to cholesterol or lecithin. The bacterial suspensions, however, had considerable neutralizing action on the active principle. After contact with a suspension of killed typhoid bacilli for 48 hours the antityphoid filtrate was 30 to 35 times less active than the original filtrate.

The Formol Titration of Lysed Cultures.

To determine whether or not the bacteriophage was able to produce a true proteolysis, a number of formol titrations were made of cultures before and after exposure to the lytic principle. For these titrations we have employed the method of formol titration described by Brown (13) for the titration of culture media. These were made with cultures after exposure to lytic principle for 8, 24, and 48 hours. The lysed cultures did not show any increase in formol titration as compared with normal control cultures of the same age kept under similar conditions. It is concluded from these titrations that the bacteriolysis caused by the bacteriophage is not a true proteolysis, but rather a simple plasmolysis of the bacterial cell without splitting of the protein molecule. This conclusion is supported by experiments now to be reported showing the persistence of antigenic properties in the filtrates of lysed cultures.

Immunization against the Lytic Principle with Filtrates of Lysed Cultures.

With the two filtrates (twentieth passage) employed in the preceding experiments, the activity and specificity of which have been described above, rabbits were immunized.

For these experiments twelve rabbits were employed. Six of them received anti-Shiga principle and six received antityphoid principle. During the course of immunization five rabbits died, three that had been injected with anti-Shiga filtrate and two injected many times with antityphoid filtrate. Others besides ourselves have noted the great toxicity of lysed culture filtrates. The Shiga filtrates were much more toxic than the typhoid filtrates. 0.1 cc. of filtrate from a lysed Shiga culture injected intravenously killed a rabbit of 1,500 to 2,000 gm. in 3 or 4 days. The autopsy picture was the same as that obtained by the injection of dysentery toxin. Filtrates of lysed typhoid cultures were much less toxic for rabbits, 2 or 3 cc. injected intravenously being generally tolerated. The same quantity of the typhoid filtrate injected subcutaneously into guinea pigs of 350 to 400 gm. was generally fatal in 6 to 8 days. The rabbits were given various quantities of the filtrates. Those receiving lysed typhoid filtrates were given five to eight intravenous injections at 5 day intervals. The total quantity of filtrate injected into each rabbit was 16 to 32 cc. Those rabbits receiving lysed Shiga filtrates were immunized in the same way and at the same intervals but the doses were much smaller, starting generally with 0.05 cc. and increasing progressively to 2.5 or 4.5 cc. for each injection, the total amount given being about 9 or 10 cc.

The sera of the rabbits were studied after five, seven, and eight injections. 8 days after the last injection the rabbits were bled to death from the carotid artery. Each serum was tested for its neutralizing action against both homologous and heterologous lytic principle. Its ability to fix complement with either filtrate as antigen was also titrated. The agglutinins for both Shiga and typhoid bacilli were titrated. The lytic properties of the washed and unwashed leucocytes of the immune animals were also studied. The presence of lytic principle in filtrates of the intestinal contents of the same animal was also investigated.

To study the lytic properties of the leucocytes, in conformity with the experiments of Lisbonne, Boulet, and Carrère (14), a leucocytic exudate was procured by the intrapleural injection of sterile bouillon into rabbits 16 hours before they were bled to death. This exudate was collected immediately after the death of the animal and divided into two parts. One portion was washed two or three times by centrifugation in physiological salt solution; the other was used without washing. The leucocytes were suspended in equal parts of salt solution and bouillon, distributed into many tubes, and inoculated with a small quantity of a young culture of typhoid or Shiga 73 dysentery bacilli; incubated at 37°C. for 48 hours; filtration done; and the lytic properties of the filtrates examined.

After bleeding the animals the intestinal contents were collected from three portions of the intestine, the duodenum, ileum, and cecum. As described above, the bacteriophage was sought for in the filtrates after incubation of the fecal suspensions.

Using the leucocytes of these immune animals we have never obtained active filtrates by the method of Lisbonne, Boulet, and Carrère. All attempts to find the bacteriophage in the intestinal contents of immunized rabbits were likewise negative. It would appear, from these experiments, that the immunization of rabbits with the lytic principle results in the disappearance of the bacteriophage from their intestines.

A study of the sera of the immunized rabbits yielded the following results.

Agglutinins.—All the sera from rabbits immunized with anti-typhoid filtrate agglutinated typhoid bacilli in rather high dilution (1:4,000 to 1:9,000). They never showed any non-specific agglutinins for the Shiga strain or the paratyphoid strains. The sera from rabbits immunized with anti-Shiga filtrate agglutinated the Shiga bacilli but only in lower dilutions (1:300 to 1:800). The agglutination tests were read after 1 hour in the water bath at 55°C. and 12 hours in the refrigerator.

Fixation of Complement.—All the immune sera fixed complement intensely in the presence of the filtrate which had served as antigen for immunization. Complement fixation occurred also in the presence of the heterologous antigen but to a much less degree. If, however, the immune sera were sufficiently diluted complement fixation occurred only in the presence of the specific antigen. These facts are easily explained as due to the antigenic complexity of filtrates of lysed cultures. It is possible that all these filtrates may possess a fraction common to the different strains of bacteria employed and that these common fractions are the cause of the apparently non-specific complement fixation in moderate dilutions of serum. All of the sera fixed complement strongly in dilutions of 1:50, 1:80, or 1:100 in the presence of the homologous antigen, whereas five or six times the amount of serum was required to fix complement in the presence of the heterologous antigen.

Antilysin.—Increasing quantities (0.1 to 1.0 cc.) of immune serum

were mixed with a constant quantity (0.1 cc.) of the homologous lytic filtrate in sterile tubes. The contents of each tube were made up to 2.0 cc. by the addition of physiological salt solution. The mixtures were incubated at 37° C. for 2 to 2½ hours. At the end of this period 0.75 cc. of a young (3 or 4 hour) bouillon culture of the homologous organism was added to each tube and after another period of 2 hours in the incubator a few drops from each tube were inoculated onto agar slants. In this manner the degree to which the lytic principle had been neutralized by the serum could be determined quite accurately. All of the immune sera were found to be more or less antilytic, although only one of the sera was found to have a titer sufficiently high to produce complete neutralization. This serum neutralized not only its homologous (antityphoid) bacteriophage but to a considerable extent neutralized also the anti-Shiga principle. However, to effect complete neutralization of the latter it was necessary to use five or six times as much serum as was required for neutralization of the homologous principle. Though there were quantitative differences all of the sera exhibited the same phenomena. In no case was a strictly specific antilysin obtained.

SUMMARY AND CONCLUSIONS.

1. The experiments here reported were carried out with a lytic principle isolated from the feces of normal rabbits.

2. It seems that bacteriophage is constantly present in the feces of caged laboratory rabbits.

3. A lytic filtrate active for Strain Shiga 73 (in a dilution of 1:1,000,000) and for the typhoid bacillus (in a dilution of 1:1,000), after twenty successive passages with Shiga bacillus was active, for its homologous strain, in a dilution of about 1:10,000,000,000 but no longer possessed any lytic activity for the typhoid strain. The same filtrate, after twenty successive passages with typhoid bacillus, was similarly active in a dilution of 1:10,000,000,000 for the typhoid strain but was only feebly active for the Shiga strain.

4. The passage of the lytic principle through collodion membranes was never observed.

5. No appreciable diminution in the activity of the filtrates was observed after exposure to cholesterol or lecithin. Killed bacterial

suspensions, however, were found to exert a considerable neutralizing effect on the active principle.

6. Formol titrations of lysed cultures lead to the conclusion that the bacteriolysis caused by the bacteriophage is not a true proteolysis but rather a simple plasmolysis of the bacterial cell and not a splitting effect of the protein molecule.

7. We have never obtained active filtrates with the leucocytes of immune animals by the method of Lisbonne, Boulet, and Carrère (14).

8. It would appear, from the experiments, that the immunization of rabbits with the lytic principle results in the disappearance of the bacteriophage from their intestines.

9. All the sera from rabbits immunized with antityphoid or anti-Shiga filtrates agglutinated respectively the typhoid or Shiga bacilli.

10. Similarly, these sera fixed complement strongly in the presence of the homologous antigen. Five or six times the amount of serum was required to fix complement in the presence of heterologous antigen.

11. All of the immune sera were found to be more or less antilytic. In no case was a strictly specific antilysin obtained.

In conclusion we wish to express our appreciation of the support of The Rockefeller Foundation, the facilities of the Department of Pathology and Bacteriology of the Johns Hopkins University, and the friendliness and counsel of Professor W. G. MacCallum, Professor J. Howard Brown, and Professor W. L. Holman.

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THE INFLUENCE OF THE SUPRARENAL GLAND ON THE THYMUS.

I. Regeneration of the Thymus Following Double Suprarenalectomy in the Rat.

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Plate 13.

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The purpose of this paper is to present experimental evidence showing the close interrelation between the thymus and suprarenal glands, our results being based upon a study of 66 doubly suprarenalectomized rats and 21 non-operated controls.

No detailed and convincing experimental literature is to be found concerning thymic enlargement following suprarenal injury or ablation. Mention of such thymic enlargement has always been reported incidentally and, in most cases, the findings are open to severe criticism.

During the last 30 years very frequent mention has been made in clinical and pathological reports of the association of a large thymus and diseased suprarenals. With the accumulation of data upon this disease, the association becomes more constant and significant. Addison (2), in his classical monograph, does not describe thymus enlargement in the disease which now bears his name, nor does Averbeck (3), who collected, in 1869, 61 cases from the literature. Star (4) was the first to call attention to thymic enlargement in Addison's disease, in the case of a girl, 17 years of age, who died suddenly, and who at autopsy showed a large thymus associated with diseased suprarenals. The more comprehensive reports of Wiesel (5), Hedinger (6), Hart (7), and Pappenheimer (8) soon after definitely established what Star had noted, the association of thymic enlargement with diseased suprarenals.

Experimentally, on the other hand, Boinet (9) and Calogero (10), using rats, and Auld (11), working with cats, have referred to thymic enlargement following suprarenalectomy. Auld reports large thymuses in four cats which died within

2 days following the removal of the second suprarenals, the first glands having been removed some months previously. While removal of one gland in the cat may induce some degree of suprarenal insufficiency, it is doubtful whether the insufficiency thus induced could have been the causative factor producing the large thymuses noted by Auld. Also there could have been no significant enlargement during the 2 days following removal of the second glands, as this time is insufficient for hyperplastic changes to manifest themselves.

Calogero's rats also died within 2 days following suprarenalectomy. Furthermore, he knew neither the age nor the life history of his animals, he gave no weights, nor did he have proper controls, so that his conclusions in regard to thymus enlargement are open to severe criticism. The apparent enlargement he reports could be attributed, for example, to age differences of his animals.

Boinet reported, among other changes, thymus enlargement in eleven rats of a series of 59 which he suprarenalectomized, many of which survived bilateral suprarenal extirpation for some weeks. Like Calogero, he knew neither the exact age nor the life history of his animals, nor was his work properly controlled; he did not give the weights or measurements of the glands which he believes were enlarged. Since he reports thymus enlargement in some animals which died within 1 and 2 days following suprarenalectomy, and, as was pointed out previously, this is physiologically improbable, his conclusions are also subject to the same criticisms that were applied to Calogero's work.

Crowe and Wislocki (12), in 1914, while studying the effects of piecemeal suprarenal ablation in dogs, reported enlargement of the thymus in four animals of a series of twenty that survived, in good condition, subtotal suprarenal extirpation for from 2 weeks to 6 months. These workers conclude that "the most striking feature at autopsy on an animal with a long-standing adrenal insufficiency is the enlargement of the mesenteric and retroperitoneal lymph glands and the solitary lymph follicles in the walls of the intestine. Not infrequently there is also an hyperplasia of the thymus." Their work was well controlled and their findings with regard to the thymus, though they had only four animals, would indicate that secondary hyperplasia of this organ will take place in dogs following subtotal suprarenal ablation.

Recently, Aub (13) and his coworkers, studying metabolic changes after suprarenalectomy, incidentally mentioned enlargement of the abdominal lymph nodes and thymus gland in their suprarenalectomized cats. Aub's animals died or were sacrificed within a few days following double suprarenalectomy. It is important to emphasize again the danger of drawing conclusions concerning hyperplasia of the thymus in moribund animals since it is definitely established that involutionary changes rapidly take place in dying animals.

Marine and Baumann (14), and Také and Marine (15), have pointed out the frequent occurrence of hypertrophy of the thymus and lymphoid tissues in rabbits which survived the removal of both glands. The present work was undertaken with the view of obtaining more specific and conclusive data on this point.

This work on the rat is the first in which thymic enlargement following suprarenalectomy has been observed and systematically studied in a large series of animals. By the use of operated and non-operated controls, and a comparison with the tables for the normal rat as compiled by Donaldson (16), we have demonstrated this change objectively. The rat is admirably adapted for such studies, in which a large number of animals is required, because a high percentage of these animals survive double suprarenalectomy and, above all, because they are highly resistant to pyogenic infection.

Methods.

All rats used were reared in the laboratory from very tame albino stock (*Mus norvegicus albinus*), obtained from the Wistar Institute. Wistar rats offer the advantage in that growth and developmental curves for the body as a whole, and for the various organs, have been tabulated.

The young are weaned at about 25 days of age and are then put on a completely adequate diet consisting of 15 parts rolled oats, 60 parts hominy, 14 parts dried meat scrap, 10 parts dried milk powder, and 1 part salt.[1] This diet is supplemented with bread and fresh milk daily until the animals are 45 days of age, after which bread and milk are given once a week; fresh vegetables are fed two or three times a week and all rats are given fresh tap water daily. During the period of active growth the rats are placed in long cages. The animals are fairly free of snuffles, and chronic pneumonia is uncommon even in 1 year old rats.

Our operative technique was standardized; both glands were removed at one sitting and after induction; ether anesthesia was always used by the open method. The operative field was prepared by shaving, and this was followed by wiping area with sponges dipped in bichloride solution. At first the operations were done through a midline upper abdominal incision, but this was later abandoned since the method of removing the glands through the back route was found more satisfactory, yielding about 85 to 90 per cent recoveries in adult rats. Food was withheld for 18 hours before operation and the rats were not fed for 12 hours after operation. For 2 days following suprarenal ablation, bread and fresh milk only were given, then the dry food mixture and vegetables were added. The operated rats were kept for about 2 weeks in individual cages, care being taken that they were warm, after which they were placed with non-operated controls in stock cages and fed like the controls. Proper preoperative preparation and diligent postoperative care, with forced feeding, have been found by all workers to be of primal importance in working with suprarenalectomized animals.

Autopsies were performed on all rats and a careful search was made for accessory suprarenal tissue. Macroscopic accessory masses in the rat are not common;

[1] Used by Professor Reid Hunt.

we have found them in about 3 per cent of our operated animals, and in rats that have not been subjected to suprarenalectomy, they are found even more infrequently. When present these visible accessory masses are usually located in the vicinity of the kidneys. The thymus glands were removed from the rats as soon after death as possible and were dissected clean of the surrounding fat, connective tissue, and lymph nodes, and weighed.

The survival of suprarenalectomized rats is explained generally on the assumption that these animals possess numerous accessory cortical rests, microscopic in size (17), located chiefly in close relation to the gonads. However, while survival may depend largely upon the presence of these microscopical accessories, we cannot but feel that additional factors come into play. A large proportion of rats and rabbits possess accessory tissue, but the rabbit is far less able to survive double suprarenalectomy than the rat, and this difference cannot be explained solely by the amount of accessory tissue present. The suprarenal glands probably stand in different quantitative relationship to the vital bodily processes in different species.

We should like to emphasize here that our experimental and control rats were kept under identical laboratory and nutritional conditions, at all times, except that the operated rats received some special care during the immediate postoperative period. The standardization of factors is extremely important in any research involving the thymus, for the gland is subject to tremendous, although to a considerable degree controllable, variation.

EXPERIMENTAL DATA.

We have summarized our experimental data in several tables. Table I shows the results with 66 doubly suprarenalectomized rats divided into two groups; namely, those dying after removal of both glands, and those which were sacrificed. Seventeen animals that died within 2 days following suprarenalectomy are tabulated. The weights of their thymuses averaged 0.155 gm., while the weights of the thymuses of seventeen animals of the same ages as given by Donaldson would average 0.202 gm. Our average is, therefore, 23 per cent below Donaldson's for animals of the same age. We also sacrificed twenty-one non-operated animals in good condition and compared their thymuses with the Wistar standards for the

same age, and found that our controls averaged in weight 17 per cent below their figures. The fact that the weights of our normal thymuses are about 17 per cent below the Wistar standards is easily explained on the basis that our animals, while kept under good physical and nutritional conditions, were kept under different conditions from the Wistar rats.

We have included the thymuses of animals dying within 2 days after suprarenalectomy with those of our normal, sacrificed controls; we have done this because 2 days is insufficient time for detectable hyperplastic or significant involutionary changes to manifest themselves.

Five rats dying within 3 to 4 days after operation had thymuses 27 per cent below the Wistar figures, while nine dying within 5 to 6 days showed thymuses 39 per cent below these figures. These low figures are due, we believe, to additional factors, arising from the circumstances that suprarenalectomized rats, succumbing gradually in the course of some days, do not take food, and consequently thymus involution due to inanition and intoxication manifests itself. It is significant that our findings are in accord with the already well established fact that the moribund state is associated with diminution in the size of the thymus, and that our figures strongly controvert the findings of several previous observers who report large thymuses in animals which died for the most part within 6 days after bilateral suprarenalectomy.

In our series, one animal, dying of progressive insufficiency 9 days after operation, had a thymus 18 per cent above the Wistar figures. We cannot explain this except on the basis of physiological variation; its thymus may have been very large at operation. Four other rats, dying 12, 14, 25, and 29 days after suprarenalectomy from progressive suprarenal insufficiency and cachexia, presented thymuses 18, 23, 49, and 43 per cent below the Wistar figures. All four lost considerable weight after operation, and the last two at death had lost respectively 38 and 37 per cent of the weight normal to rats of their age.

We pass now to a consideration of the thirty animals which were killed by the injection of standard typhoid vaccine in the course of some immunological experiments, or were sacrificed from 9 to 207

days after suprarenalectomy (Table I). All animals included in this series which were killed with standard typhoid vaccine died within 6 hours after its administration and the thymus weights could not have been appreciably affected during this time interval. One is immediately impressed by the remarkable difference shown

TABLE I.

	Length of time after suprarenalectomy.	Cause of death.	No. of rats.	Averaged thymus weights, suprarenalectomized rats, at autopsy.	Averaged thymus weights, normal rats, same age (Donaldson).	Percentage differences between thymuses of suprarenalectomized and normal (Donaldson) rats.
	days			*gm.*	*gm.*	*per cent*
	1–2	Acute postoperative.	17	0.155	0.202	−23
	3–4	" "	5	0.177	0.244	−27
	5–6	" "	9	0.123	0.203	−39
Died.	9	Progressive suprarenal insufficiency.	1	0.220	0.186	+18
	12	" "	1	0.141	0.172	−18
	14	" "	1	0.169	0.220	−23
	25	" "	1	0.103	0.203	−49
	29	" "	1	0.113	0.199	−43
	9	Vaccine.	2	0.200	0.180	+11
	12	"	1	0.235	0.175	+34
	18	"	2	0.297	0.162	+83
	22	Died after a struggle.	2	0.334	0.148	+125
Killed by	25	Vaccine.	1	0.235	0.170	+38
vaccine	29	Exhaustion from fighting.	1	0.375	0.288	+30
or sacri-	30–35	Sacrificed.	5	0.326	0.150	+117
ficed.	41	"	3	0.232	0.141	+65
	52	"	4	0.209	0.155	+34
	65–75	"	5	0.200	0.142	+40
	170	"	1	0.160	0.085	+88
	207	"	3	0.110	0.086	+16

when their thymus weights are compared with Donaldson's standards for the same ages in normal rats. All thirty rats had thymuses above the Wistar standards. Two of these thirty were killed by vaccine 9 days after suprarenalectomy; they were in good condition at the time of injection and probably would have survived indefinitely. Their thymuses averaged 11 per cent above the Wistar standards.

Another was killed by vaccine at 12 and still another 18 days after suprarenalectomy, and their thymuses were 34 and 83 per cent respectively above the Wistar figures. Two others died shortly after blood had been taken from the tail, as result apparently of struggling; their thymuses averaged 125 per cent above the Wistar standards. One rat was killed by vaccine 25 days after suprarenalectomy, while a second died 29 days after suprarenalectomy from exhaustion incident to fighting with another animal. Their thymuses were 38 and 30 per cent respectively above the normal weight. Five rats, sacrificed 30 to 35 days after operation, had thymuses which averaged 117 per cent above the normal. 41 days after operation 3 rats had thymuses averaging +65 per cent; at 52 days, 4 had thymuses averaging +34 per cent; at 65 to 75 days, 5 had thymuses averaging +40 per cent; at 170 days, 1 had a thymus which was +88 per cent; and at 207 days, 3 had thymuses averaging +16 per cent.

When our figures are corrected on the basis of a normal thymus weight about 20 per cent below Donaldson's standards, the changes become even more significant, the actual increase in size of the thymuses of these thirty suprarenalectomized rats being about one-fifth more than the apparent increase.

Table II presents some individual data, summarized from the protocols. It has to do with twenty-seven of the thirty rats that were sacrificed or killed by vaccine. Analysis of this table yields the following information.

(*1*) *Age.*—One animal was operated on during the 2nd month of life, four during the 5th month, and twenty-two during the 6th and 7th months. Thus twenty-six of the twenty-seven were operated upon after thymus involution normally would have already begun, and of these, twenty-two when involution was probably already well established.

(*2*) *Gain or Loss in Weight Following Operation.*—Twenty-one of the twenty-seven were killed or sacrificed within 60 days after operation. Of these, eleven lost from 1 to 14 per cent of their original weight at operation, one did not change in weight, while nine gained from 1 to 22 per cent. The other six rats, sacrificed after 60 days, gained in weight. The significant feature is that 52 per cent of the

animals surviving double suprarenalectomy in good condition up to 60 days lost weight in spite of the fact that they were eating well, frequently much more than their controls, and had not attained

TABLE II.

Rat No.	Sex.	Age at operation.	Length of time after operation.	Weight at operation.	Weight at death.	Percentage gain or loss in weight since operation.	Percentage difference in weight at death between the suprarenalectomized rats and normal (Donaldson) rats of same ages.	Percentage differences between thymuses of suprarenalectomized and normal (Donaldson) rats.
		days	*days*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
RW 12-2	M.	162	9	262	230	−12	−1	+5
RW 12-1	"	184	9	213	200	−6	−18	+18
RW 25-2	F.	175	12	149	140	−6	−31	+34
RW 31-3	"	184	18	212	202	−4	−3	+135
RW 31-4	"	184	18	165	165	0	−21	+33
RW 16-3	"	196	22	200	185	−7	−13	+111
RW 16-2	"	196	22	180	155	−14	−27	+140
RW 30-2	"	168	25	176	170	−3	−17	+38
RW 3-2	M.	58	29	150	165	+10	+13	+30
RW 33-1	F.	178	31	200	245	+22	+16	+130
RW 33-4	"	178	31	210	235	+12	+11	+121
RW 33-2	"	178	34	140	135	−3	−36	+30
RW 33-3	"	178	34	190	205	+8	−3	+167
RW 20-3	"	201	34	171	185	+8	−15	+131
RW 30-3	"	168	41	188	210	+11	0	+63
RW 30-4	"	168	41	195	175	−10	−17	+63
RW 20-4	"	223	41	197	200	+1	−10	+65
RW 29-3	M.	158	51	255	242	−5	−4	+59
RW 29-1	"	158	52	240	245	+2	−3	+9
RW 29-4	"	158	52	200	185	−7	−26	+50
RW 30-1	"	158	52	225	245	+9	−3	+19
RW 16-1	F.	196	65	215	295	+37	+32	+74
RW 17-1	"	143	67	175	225	+28	+6	+45
RW 15-1	M.	133	72	181	235	+30	−6	+28
RW 14-4	"	133	73	192	230	+19	−8	+12
RW 12-4	F.	172	77	178	215	+20	−3	+55
RW 9-3	"	97	170	186	255	+37	+12	+88

their maximum weights at the time of operation. It has been pointed out by the early observers that animals surviving double suprarenalectomy for a week or more frequently lose weight rapidly and this loss of weight is due largely to the rapid disappearance of fat. This

fact lends clinical support in the case of the rat to the findings of Marine and Baumann (14, 18) and Scott (19), that removal or crippling the suprarenal glands in rabbits and cats frequently causes a disturbance in metabolism characterized by a prolonged rise in heat production. In this connection it is important to point out that while cachectic and marasmic states ordinarily result in involution of the thymus, this organ enlarges after suprarenalectomy in spite of the loss in weight, even when the animal is much below the average weight for its age. We know of no clinical conditions except Addison's and Graves' diseases, and occasional instances of status lymphaticus, in which the thymus may enlarge in spite of a considerable loss in weight.

(*3*) *Curve of Thymus Enlargement.*—The last column of Table II would seem to indicate that thymic enlargement following suprarenalectomy describes a curve. As has already been noted (Table I) rats dying in from 5 to 6 days after bilateral suprarenal ablation have thymuses much below the standard weight, while those surviving operation 5 to 6 days in good condition, we believe possess thymuses of normal size or even slightly enlarged. About this time the stimulus for the thymic enlargement exerts a detectable effect, the organ reaching its maximum size between the 3rd and 5th weeks after suprarenal ablation. After the height of the curve is attained, there is a gradual decline in the size of the gland, but for months after operation it still does not approach that of the normal age control. These changes may be interpreted on the basis that an active secondary hyperplasia in the involuted thymus occurs soon after bilateral suprarenal ablation, and that when the gland has regenerated to that size which existing physiological and pathological conditions permit, and then as compensation begins to influence it, normal involution, though manifesting itself, is markedly delayed.

Table III shows the results in ten suprarenalectomized rats which lived from 18 to 67 days after operation. Each rat is compared with a control of the same, or approximately the same age. The data bring out clearly the differences in the thymuses.

TABLE III.

Rat No.	Sex.	Age at death.	Weight at death.	Cause of death.	Length of time after suprarenalectomy.	Weight of thymus.	Percentage differences between thymuses of suprarenalectomized and control rats.
		days	*gm.*		*days*	*gm.*	*per cent*
RW 31-3	F.	202	202	Vaccine.	18	0.380	+162
RW 20-2	"	199	155	Acute postoperative.	2	0.145	
RW 16-3	"	218	185	Died after a struggle.	22	0.313	+71
RW 29-2	M.	209	285	Sacrificed.	Control.	0.174	
RW 16-2	F.	218	155	Died after a struggle.	22	0.355	+386
RW 37-1	M.	211	210	Sacrificed.	Control.	0.073	
RW 33-1	F.	209	245	"	31	0.359	+174
RW 32-1	M.	209	245	"	Control.	0.131	
RW 33-4	F.	209	235	"	31	0.345	+130
RW 36-4	M.	210	285	"	Control.	0.150	
RW 33-3	F.	212	205	"	34	0.415	+315
RW 32-2	M.	212	210	"	Control.	0.100	
RW 30-4	F.	209	175	"	41	0.255	+88
RW 34-2	"	209	185	"	Control.	0.135	
RW 29-3	M.	209	242	"	51	0.248	+72
RW 32-3	"	209	250	"	Control.	0.144	
RW 16-1	F.	261	295	"	65	0.200	+78
RW 31-1	"	264	202	Acute postoperative.	2	0.112	
RW 17-1	M.	210	225	Sacrificed.	67	0.225	+58
RW 17-4	"	210	245	"	Control.	0.142	

Unilateral Suprarenalectomy.

We studied five unilaterally suprarenalectomized rats and did not observe in any of these the definite enlargement of the thymus that occurs after bilateral ablation, probably because little if any suprarenal insufficiency had been induced.

Sex Differences.

Of the twenty-five animals of Table II, which were killed or sacrificed 12 days after operation, seven were males Their thymuses examined 29, 51, 52, 52, 52, 72, and 73 days after bilateral suprarenal ablation were 30, 59, 9, 50, 19, 28, and 12 per cent above the Wistar standards. On the whole, these thymuses are not as large as those of females of corresponding ages. We have not, however, sufficient data to establish this fact conclusively.

Pregnancy.

A previous pregnancy does not prevent the thymus from enlarging after suprarenalectomy. Seven of the eighteen females which were sacrificed had gone through a pregnancy before operation. Two were suprarenalectomized 2 weeks after casting litters and were killed by vaccine 18 days after operation. Their thymuses were 135 and 33 per cent above the Wistar standards. Two went through pregnancy 4 months before ablation of the glands and were autopsied 22 days after operation, their thymuses being 111 and 140 per cent above the Wistar standards. Three others were pregnant at various times before operation and were sacrificed 41, 65, and 170 days after suprarenalectomy. They also had large thymuses.

Chronic Lung Infection; Incomplete Removal of Suprarenals.

Animals presenting chronic lung infections were excluded from our series, since as is well known, pathological thymic involutions are rapidly induced by these infections. Nevertheless, we have seen large thymuses in a few bilaterally suprarenalectomized rats with chronic pulmonary infections. It would seem from this that the stimulus for hypertrophy and hyperplasia of the thymus following suprarenal ablation is so marked that involutionary changes may at times be counteracted.

The suprarenals are capable of undergoing rapid regeneration and when both glands are incompletely removed, the residual fragments may within a month attain a size even greater than that of the original glands. Rats examined a few months after incomplete suprarenalectomy and presenting regenerated suprarenal masses on both sides,

show no enlargement of the thymus. If in such instances there had been thymus hyperplasia soon after operation, regression must have rapidly taken place.

Histology.

The tissues were studied histologically following Orth's fixation, paraffin embedding, and staining with hematoxylin and eosin.

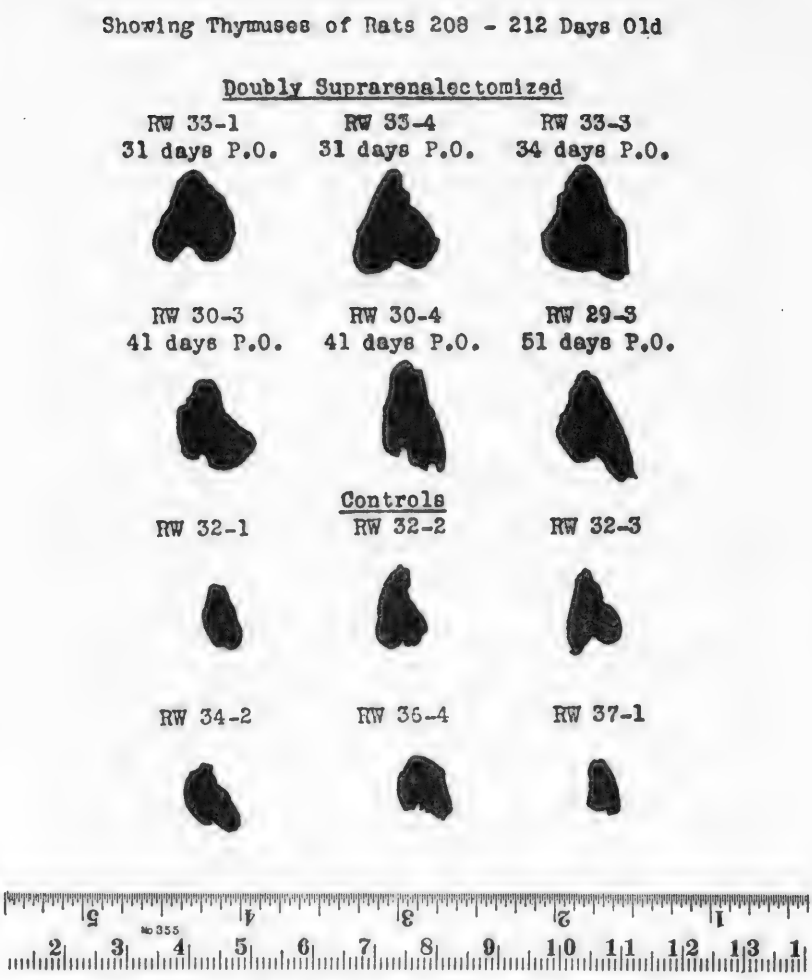
Striking differences are apparent even in the gross between the regenerated thymuses of mature rats 30 or 40 days after suprarenalectomy and those of their non-operated age controls.

The glands of the former are large, white, and fleshy, while those of the latter are small, yellowish in color, and show fine connective tissue septa penetrating the organ. On histological examination the differences become even more apparent; the hyperplastic glands show large and closely apposed lobules, which are separated only by thin fibrous tissue septa. The bulging lobules are differentiated into distinct cortical and medullary zones, the cortical zones being thick and packed with closely aggregated small thymic cells, which occasionally show mitotic figures. The medulla is prominent, contains a few small thymic cells, a moderate number of reticular cells, and well formed Hassall's corpuscles. A number of our sections also show well formed, round, or irregularly alveolated, small and large duct spaces, lined by single or double layers of epithelium. Histologically, the large regenerated thymuses of mature suprarenalectomized rats resemble closely the growing thymuses of normal young animals, 50 to 70 days of age. One of the unmistakable evidences, however, that these large thymuses have regenerated, is the thickened arteries that are present in an otherwise plump, active gland.

Since our series includes thymuses examined from 9 to 170 days after suprarenalectomy, we have been able to trace the various steps in the secondary hyperplasia which follows suprarenal extirpation, and in the delayed involution that ensues after the regeneration of the thymus has become maximal.

The thymuses of the mature non-operated rats show involutionary processes which are manifested by a diminution in the amount of parenchymal substance, the appearance of a relatively abundant stroma, broadening of the septa, and the development of variable amounts of adipose tissue about the larger blood vessels. The vessels themselves show thickening of their coats. The differentiation between cortex and medulla is still present but both zones are reduced in size. In the more advanced involutions the lobules appear irregular in shape and reduced in size, the connective tissue about the blood vessels is more prominent and hyalinized, and the demarcation between cortex and medulla becomes obscure.

The thymuses of rats dying 5 to 6 days after operation show extensive disintegration of the small thymic cells, and blurring of the markings between cortex and medulla. These changes are dependent undoubtedly upon the toxemia and



TEXT-FIG. 1. Showing thymuses of twelve rats 208 to 212 days of age, six of which were suprarenalectomized 31 to 52 days before being sacrificed. One-tenth less than actual size.

acute inanition which generally are associated with the moribund state, and account for the marked diminution in weight of these thymuses below the Wistar standards.

Text-fig. 1 and Figs. 1 to 3 illustrate some of the changes described. The text-figure shows twelve thymuses of rats 208 to 212 days of age, six of which had been suprarenalectomized 31 to 52 days before being sacrificed, and their thymuses are compared with those of non-operated controls. Fig. 1 is a photomicrograph of the thymus of Rat RW 33-3, showing the marked regeneration and secondary hyperplasia. Fig. 2 shows the thymus of an age control (No. RW 37-1), with well established involution. Fig. 3 is a photomicrograph of the thymus of a rat 51 days of age, showing the striking resemblance between a normal growing and a secondarily regenerated thymus.

SUMMARY AND DISCUSSION.

Experimental evidence has been presented showing that the thymus gland of the rat enlarges rapidly in those animals surviving double suprarenalectomy. The enlargement has been observed to follow bilateral ablation with such constancy that it may be said to occur in all rats which survive double suprarenalectomy in good condition for from 3 to 5 weeks. The thymus enlarges even though the animal loses some weight after operation. Our data, though inconclusive, would seem to indicate a sex difference, the secondary hyperplasia of the thymus being more marked in the female than in the male. This difference, we believe, may shed some light on the mechanism of thymus enlargement following double suprarenalectomy. In the rat there is a definite sex difference in the weight of the suprarenals (20), the glands being much larger in the female than in the male. This weight difference becomes greater as the rat grows, and it appears at an early period of life; indeed, it is obvious at about 35 days of age, in spite of the fact that sexual maturity is seldom attained before 60 to 90 days. This difference in the suprarenals is independent of pregnancy. It is highly probable that the suprarenal glands stand in closer relation to the sex organs of the females than in the male, and that removal of these glands disrupts

the sex interrelationships between gonads, suprarenals, and thymus, this disturbance being more definitely expressed in the female than in the male.

The great majority of the rats were operated on when they were between 6 and 7 months of age; that is, after thymus involution would normally have set in. Thymuses examined when they had reached their largest size resembled both grossly and microscopically the growing thymuses of much younger animals, the only evidence of previous involutionary change being the presence of thickening of the vessels. We assume, therefore, from indirect, but statistical data, that regeneration of the involuted thymus must have taken place. The regeneration following bilateral ablation describes a curve which reaches its height between the 3rd and 5th weeks after operation. After the height of enlargement is attained, there is a gradual decline in the size of the gland, but involutionary changes may be retarded for months. Pregnancy prior to suprarenalectomy does not prevent thymus regeneration following this operation. Thymic enlargement does not ordinarily take place following a unilateral ablation. Severe chronic infections may sometimes bring about such a pronounced pathological involution of the thymus that the hyperplasia may be prevented.

There can as yet be only speculation as to the mechanism involved in the regeneration of the thymus which follows suprarenalectomy. This secondary thymic hyperplasia may be one manifestation of the generalized lymphoid hyperplasia that follows sublethal but sufficient suprarenal injury, and which is characterized by the appearance of prominent lymphoid foci in the various organs, particularly in the thyroid, a generalized hyperplasia of the lymph nodes and bone marrow, and the appearance of a lymphocytosis in the blood. The evidence at present available would suggest that both the thymic and general lymphoid hyperplasia may be effected through the gonads and parasex tissues and that the thymus especially regenerates because of a disruption of the interrelations between the interrenal gland, the gonads, and the thymus.

The close association between the gonads and the suprarenals (21) is well known. The latter undergo enlargement in pregnancy, ovulation, and castration, and the ovaries hypertrophy after sup-

rarenalectomy. Tumors of the suprarenals are associated frequently with sex perversion and precocity both in the male and female. The interrelation between the gonads and thymus is classical, castration before puberty delaying thymus involution for a long time.

A number of diseases present thymus enlargement among the major pathological findings at autopsy. The most outstanding of these are Addison's disease, status lymphaticus, and Graves' disease. It is now generally recognized that the suprarenal glands play an important rôle in the etiology both of Addison's disease and status lymphaticus. Whether the interrenal or the chromophil system is primarily involved or whether disturbances in function of both systems are at the basis of these diseases is still undetermined. The newer evidence, including our own work on lymphoid regeneration following suprarenalectomy supports the view that the lymphoid hyperplasia occurring in both Addison's disease and status lymphaticus is dependent upon insufficiency of the interrenal system (suprarenal cortex). While anatomical studies have not disclosed any constant lesions in the suprarenals in Graves' disease, evidence is being accumulated which would indicate that functionally these glands also play an important if not a primary rôle in the production of the clinical syndrome of Graves' disease (14, 18, 19). We are of the belief that the persistence of the thymus which occurs in status lymphaticus, and the regeneration which occurs in Addison's and Graves' diseases, are brought about by the same disturbances in glandular interrelations which bring about regeneration of the thymus in the experimental animal after suprarenalectomy.

CONCLUSIONS.

1. Secondary hyperplasia of the thymus resulting in enlargement of the organ follows, with great constancy, double suprarenalectomy in adult rats.

2. The height of the curve of this enlargement is reached in from 3 to 5 weeks after bilateral suprarenal ablation.

3. The enlargement seems to take place more readily in the female than in the male, is not prevented by a previous pregnancy, and occurs even when the animal has lost weight after operation.

4. The enlargement of the thymus following double suprarenalectomy is believed to result from a disturbance of the interrelations between the gonads, the thymus, and the interrenal gland (suprarenal cortex).

5. A possible relationship between the enlargement of the thymus occurring in Addison's disease, Graves' disease, status thymicolymphaticus, and that following double suprarenalectomy is suggested.

I wish to express my sincere appreciation of the suggestive and stimulating criticism of Dr. Marine, which has made this work possible.

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EXPLANATION OF PLATE 13.

FIG. 1. Photomicrograph of the thymus of Rat RW 33-3, 212 days of age, showing marked regeneration and secondary hyperplasia. Hematoxylin and eosin. × 60.

FIG. 2. Photomicrograph of the thymus of Rat RW 37-1, non-operated control 211 days of age, showing well established involution. Hematoxylin and eosin. × 60.

FIG. 3. Photomicrograph of an actively growing thymus of a rat 51 days of age. Hematoxylin and eosin. × 60.

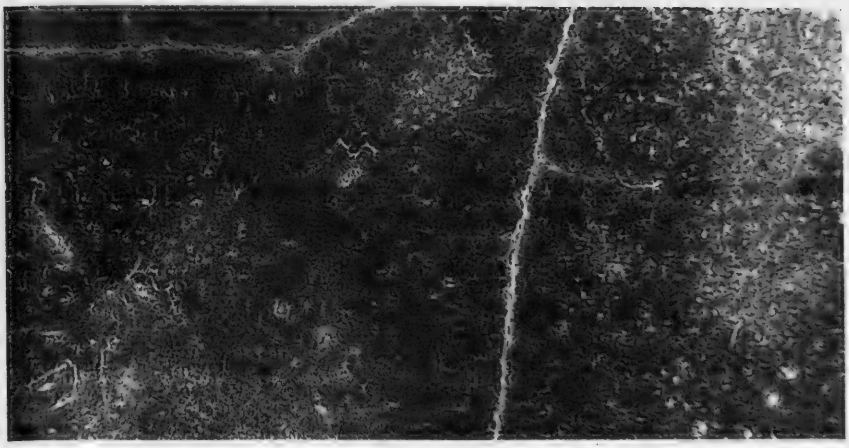

FIG. 1.

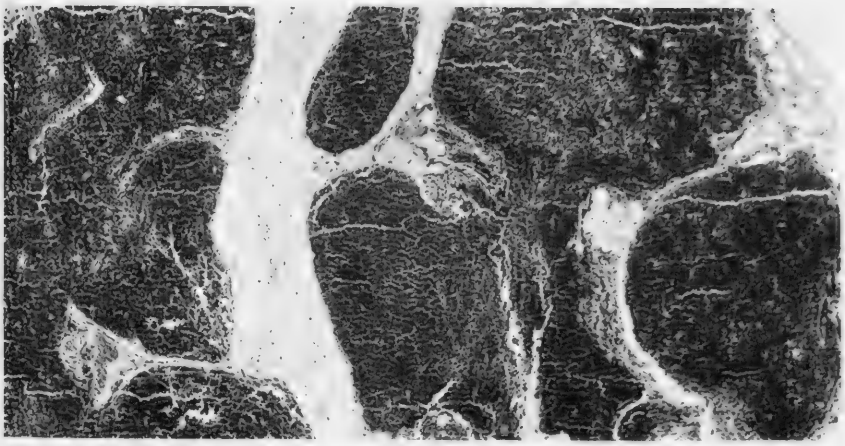

FIG. 2.

FIG. 3.

(Jaffe: Influence of suprarenal gland on thymus. I.)

THE CHEMICAL NATURE OF RESIDUE ANTIGEN PREPARED FROM YEAST.

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(Received for publication, April 29, 1924.)

It has been shown, in previous communications (1, 2), that extracts of a great many different species of bacteria (tubercle bacilli, pneumococci, *Bacillus influenzæ*, *Staphylococcus pyogenes aureus*, streptococci, and meningococci), prepared in such a way as to be as free as possible from coagulable proteins and acid-insoluble proteins, contain substances which specifically precipitate with homologous immune sera, produce delayed skin reactions of the tuberculin type in infected animals, and do not induce antibody formation on repeated and energetic administration to rabbits. Since these properties seem to represent the antigenic haptophore group of the bacteria, and promise to possess considerable importance in connection with the pathology of infection, because of their violent reactions with the sensitized animal (2) it has appeared essential to study their chemical properties and constitution in greater detail. As a result of a series of chemical analyses of preparations from various bacterial sources, reported by one of the writers with Wayman and Zinsser (3), two points were established: (1) that the material as prepared was very low in nitrogen, and, therefore, probably contained a considerable quantity of a non-nitrogenous substance; and (2) that the small yield, coupled with the colloidal nature of the substance and the presence of several impurities, made the task of purifying and establishing the nature of the active compound almost impossible from bacterial sources at our command. Because it had been possible to obtain preparations having similar biological and serological properties from every bacterial species with which the attempt had been made, it seemed possible

that other varieties of microorganisms, such as the yeasts, might serve equally well, and that perhaps by using a commercial bread yeast or brewer's yeast enough residue could be obtained for further study. It did not seem impossible that facts gleaned from the study of such material could be applied to further investigation of the smaller quantities obtained from bacteria.

Accordingly, rabbits were injected intraperitoneally first with dead, later with live yeast, using a strain isolated in the spring of 1923 from Fleischmann's compressed yeast.

After several injections at intervals of 5 to 6 days, rabbits were bled and the serum tested for agglutinating properties against suspensions of the yeast. Agglutination was definite but slow up to a serum dilution of about 1:100, or a little better, but did not increase materially after several further injections. The comparatively low titer may be due in part to the large size of the yeast cells, causing them to settle rapidly out of suspension, and in part, perhaps, to an inherent antigenic weakness on the part of yeast. We have found that some rabbits will yield a much higher titer serum than others, and have had one in our series which failed to produce even a trace of antibody when injected in parallel with others which had reached a fairly high level. Normal rabbit sera, used as controls, gave, of course, no agglutination with the yeast suspension.

The next step consisted in the preparation of an extract of yeast cells by a method similar to that used in the preparation of bacterial residue antigens.

For this purpose the "starch-free" grade of Fleischmann's yeast was used. A small quantity was extracted with saline, after bringing to pH 9 to 10 with NaOH, by shaking for several hours. The material was centrifugalized, acidified slightly with acetic acid, filtered, neutralized, and precipitated with alcohol. The precipitate, taken up in saline, gave a good precipitin test (ring) with the yeast serum, but not with the serum of normal controls.

Material prepared in this way was low in nitrogen, having between 1.0 and 2.0 per cent, and gave a strong Molisch test. The yield, however, was small, most of the yeast cells remained viable after extraction, and probably much of the specific substance was lost.

About this time Heidelberger and Avery (4) reported their work on the specific precipitable substance of pneumococcus, a substance almost certainly identical with Zinsser's residue antigens. Their results were similar to those already obtained by us in so far that the

nitrogen was low (1.2 per cent) and that the bulk of their preparation was carbohydrate in nature. Because of the fact that the specific properties of their preparation increased as the nitrogen content diminished down to their minimum figure, they believed that the active material was non-nitrogenous and that the carbohydrate was the specific substance. While their work was convincing, we did not feel that the presence of two nitrogenous bodies, one inactive, the other, represented by the remaining 1.2 per cent of nitrogen, active, had been completely excluded. If such a supposition were correct, their material would contain about 90 per cent carbohydrate, and 10 per cent of some material with roughly the same nitrogen content as protein, the two being extremely difficult to separate by means of the physical methods of fractional precipitation with alcohol, salting out with $(NH_4)_2SO_4$ and dialysis, which had been used. Such a nitrogenous compound, when pure, should give a ring test up to a dilution of 1:30,000,000. However, in view of their work, and of the invariably strong Molisch test given by residue antigen preparations from other bacterial sources, and now from yeast, it seemed that one must attach some significance to the complex carbohydrate material in these extracts.

Several such compounds have been isolated from yeasts, and reported in the literature. One, particularly, the so called yeast gum, described by Salkowski (5), and more fully by Oshima (6), and by Meigen and Spreng (7), seemed to merit closer investigation. It was extracted by them from yeast with hot potassium hydroxide, precipitated by Fehling's solution as the copper compound, and freed from copper by solution in dilute HCl and reprecipitation with alcohol. It is described as being free from nitrogen and phosphorus, and hydrolizing with acid to dextrose and mannose. It had attracted attention mainly because of its frequent presence in invertase preparations.

We found at once that our yeast residue antigen preparations gave a precipitate with Fehling's solution, and that this precipitate carried with it the specific precipitable substance of the yeast. The preparation of yeast gum by a method practically identical with Salkowski's was therefore undertaken.

Preparation of Yeast Gum.

1 pound of Fleischmann's starch-free yeast was suspended in 2 liters of 1 per cent NaOH, heated in a large evaporating dish for ½ hour on the water bath, neutralized with glacial acetic acid to faint acidity to litmus, and filtered through paper. To the filtrate, Fehling's solution was added until a precipitate began to separate, requiring between 200 and 300 cc. of the reagent. The bulky precipitate, which clumps and settles out as a friable gum, was washed two or three times by pressing out in water, ground in a mortar with water to which strong HCl was added drop by drop, until the blue precipitate had completely dissolved, and the solution was somewhat green. Three volumes of alcohol were then added, and the mixture allowed to stand for some hours, until the precipitate had settled. The latter was again dissolved in water, a few drops more of HCl added, and reprecipitated by alcohol. The precipitate, after settling out, was filtered on a suction filter, washed with alcohol and ether, and dried. It still contained a trace of copper, gave a non-specific ring, probably because of the copper, in a dilution of 1:100, and specific ring tests with anti-yeast sera, at 1:1,000 and 1:10,000. The yield was about 10 gm.

By minor modifications of this method, using NaOH up to 2 per cent, and heating up to 1 hour, similar yields were obtained, until in all some 60 gm. of the yeast gum had been collected, giving a good specific ring test up to 1:10,000 in 15 to 20 minutes, and 1:100,000 in 2 to 3 hours.

At this stage the work was interrupted in the spring of 1923, to be resumed in the fall. It was necessary at this time to reisolate a strain of yeast for immunization purposes, and to inject new rabbits with the strain thus obtained. The serum obtained precipitated the yeast gum prepared in the spring in the same dilution previously obtained (1:100,000 in 3 hours), but it proved impossible to prepare a yeast gum by Salkowski's method from yeast then obtainable which gave any precipitate whatever with the immune sera. The gum obtained although apparently similar to the earlier material was completely inactive serologically. After many fruitless attempts to obtain an active material by the method used so satisfactorily a few months earlier, we were forced to abandon that method of preparation. Because of the ease with which active gum was at first made, with several different concentrations of NaOH, and periods of heating, the most obvious explanation for the difficulty, which, however, we have not been able to confirm, is that either the commercial

strain of yeast, or the method of manufacture, changed during the summer, and that the specific material of the product now marketed is more susceptible to destruction by hot alkali than that which had been obtainable a year previously. Whatever the explanation, the 60 gm. of material prepared by the hot alkali method have retained their activity, and have served for a considerable amount of work on purification which will be described below.

The yeast obtainable through the present winter has agglutinated with the immune sera just as well as that used earlier. Residue antigens prepared by Zinsser's original method have shown good specific rings with the sera, but for quantity production it has been necessary to modify the original method of preparation to the extent of avoiding, as far as possible, treatment with hot alkali. The method finally adopted, after a number of experiments in various directions, is as follows:

Fleischmann's compressed yeast is autoclaved without the addition of water, at 15 pounds for from 2 to 3 hours. This is best carried out in tall beakers, filled not over one-third full. At the end of this stage, the yeast has become semifluid, like thick cream. A little solid NaCl, 10 to 15 gm. to a pound of yeast, is stirred into the mass, and it is poured into boiling water, 2 liters to a pound, covered, and allowed to boil slowly for about 2 hours. After partial cooling, the suspension is poured into tall glass jars, and allowed to settle overnight. The supernatant is siphoned off, the sediment filtered by suction, and the combined supernatant and filtrate run through a Sharpless centrifuge.

Fehling's solution, in the proportion of 150 to 200 cc. to a liter of extract is now added, and the mixture warmed in a double boiler for a few minutes until the precipitate begins to separate and contract. The supernatant is carefully decanted, the gummy precipitate pressed together with a spatula, and finally washed two or three times by pressing out in water.

From this point the method is exactly as described originally, dissolving in dilute HCl, precipitating with alcohol, and again dissolving and precipitating if desired.

The yield is only about one-half that previously obtained; namely, 5 gm. from a pound of material.

These two preparations were about equally active as shown by the precipitin test against an anti-yeast rabbit serum. Analyses showed the following.

	Hot NaOH preparation.	Autoclave preparation.
	per cent	*per cent*
Total N	0.51	1.21
" P	0.42	0.61
Reducing substances after acid hydrolysis, calculated as glucose	84	
Specific rotation	+83°	

Further Purification of Hot NaOH Preparation.

25 gm. of this preparation were dissolved in 500 cc. of water, centrifuged from a small amount of fine white precipitate, during which process some material was lost by a broken tube, and about 400 cc. of alcohol added slowly, with stirring until a definite permanent precipitate had formed. This was allowed to settle overnight, the supernatant poured off, and the precipitate washed with alcohol and ether, and dried. Marked Precipitate I.

To the supernatant fluid 200 cc. more of alcohol was added. A fine, colloidal precipitate was formed, which, on standing, settled out as a sticky syrup on the bottom and sides of the beaker. It was washed with alcohol and ether, and dried, and marked Precipitate II.

Further addition of alcohol to the supernatant from Precipitate II produced only a minimal amount of precipitate, and was not investigated further.

	Weight.	Total N.	Titer.
	gm.	*per cent*	
Precipitate I	3.5	0.73	1:100,000 in 1½ hrs.
" II	16.2	0.19	1:100,000 " 1½ "

Precipitate I was fractionated further by dissolving in water and adding alcohol up to the first visible permanent precipitate, and allowing to stand overnight. This precipitate (No. IA) was dried, and the filtrate precipitated further with alcohol, leading to a syrupy material like Precipitate II. Precipitate IA was a dark-colored, granular material. It contained 1.65 per cent N and gave a ring test at a dilution of 1:100,000 in 3 hours.

An aqueous solution of Precipitate II gave no precipitate with the following reagents: tannic acid, picric acid, phosphotungstic acid, $MgSO_4$ (saturated), $(NH_4)_2SO_4$ (saturated), lead acetate, mercuric chloride, and mercuric cyanide.

A further attempt at purification by dialysis through a fish bladder against running water for 4 days resulted in a practically quantitative recovery of the amount used, with no change in precipitin titer or nitrogen content.

Further Purification of Autoclave Preparation.

60 gm. of material, resulting from 12 pounds of compressed yeast, were dissolved in about 700 cc. of water, a fine white undissolved residue was centrifuged off, dried, and saved as "insoluble material." The clear solution was fractionated by successive additions of alcohol, as in the preceding preparation, four fractions being separated, the first with about one-half volume, the last with about three volumes of alcohol. After removing the fourth precipitate, the supernatant was evaporated to dryness and the residue reserved. A summary of the separation follows.

Fraction.	Volumes alcohol to precipitate.	Weight.	N	P	Titer.
		gm.	*per cent*	*per cent*	
Insoluble.....		5.2	1.59	0.72	
Precipitate I....	One-half.	8.8	2.52	1.23	Trace in 1:200,000 in 2 hrs.
" II....	One.	30.2	0.69	0.47	" " 1:400,000 " 2 "*
" III...	" and one-half.	10.8	0.67	0.49	" " 1:400,000 " 2 "*
" IV....	Three.	5.4	0.96	0.48	+ " 1:200,000 " 2 "*
Residue.		3.3	0.88	0.42	

* Trace in 1:800,000 in 6 hours.

Reserving a small amount of Precipitates II and III, the remainders were mixed together, dissolved in 2,000 cc. of water, and reprecipitated by Fehling's solution. About 30 gm. of the gum were used for this purpose, and after washing the precipitate, dissolving, and reprecipitating twice with three volumes of alcohol, it was dissolved in water containing somewhat more HCl than that necessary to give the solution a clear, yellowish green color, and poured into seven volumes of alcohol. After standing overnight, it was filtered off and dried. Yield—20 gm., which still contains a faint trace of copper. The precipitin titer is unchanged, 1:400,000 in about 2 hours.

A small trial lot of this preparation showed that the specific reaction was unchanged upon treatment for as long as 15 hours with N HCl and N NaOH at room temperature, although a few minutes of heating with either reagent destroys it. Accordingly, the remainder of the 20 gm. was dissolved in 400 cc. of N HCl, 2,800 cc. of alcohol added, and the precipitate allowed to settle overnight. The precipitate was filtered off, dissolved in 400 cc. of water, and again thrown down by seven volumes of alcohol, and finally dissolved in 400 cc. of N NaOH, alcohol added as before, and the precipitate, after settling, taken up in 150 cc. of water. To this solution was added six volumes of glacial acetic acid, the precipitate separated as rapidly as possible by centrifuging and washing with alcohol, and dried. Yield—12 gm.

This material gives a definite ring at 1:400,000 in ½ hour, but the 1:800,000 tube remains negative after several hours. It contains 0.56 per cent N, 0.028 per cent amino nitrogen by Folin's colorimetric method (8) before hydrolysis, and 0.089 per cent amino nitrogen after heating with 50 per cent HCl for 5 hours in a sealed tube at about 105°. The latter figure is little more than an approximation, because of the necessity for evaporating the HCl and decolorizing the residue with charcoal. The phosphorus content was still 0.48 per cent. Its specific rotation was +90°.

The description of the products obtained in the attempted purification of the starch gum, makes it apparent that we must face the same difficulty of interpretation of results as outlined above in the discussion of Heidelberger and Avery's pneumococcus work. Is the yeast gum the substance which carries the specific properties of the yeast residue antigen, and does its molecule contain a low percentage of nitrogen and phosphorus; or are the latter two elements combined in some other substance which it has been impossible to separate from the gum merely through failure to discover a suitable method? While we admit that the point is not firmly established experimentally, we are inclined to adhere to the former view, because of the following considerations.

Referring to the fractional precipitation of the hot NaOH preparation by alcohol, Precipitate IA contains 1.65 per cent N, while Precipitate II contains only 0.19 per cent. Hence, if the specific property were following the nitrogen, No. IA should be nearly ten times as active as No. II, which is not the case. As a matter of fact, there is very little difference between the two, as would necessarily be the case if the yeast gum were the active agent. Precipitate IA probably contains about 85 per cent gum, against 97 to 98 per cent in Precipitate II, a difference insufficient to recognize by the precipitin test. Moreover, we have carried out numerous experiments with extractions of various sorts, cold acid and alkali, autolysis, etc., and have never obtained a preparation which would give a precipitin test without also giving a gummy precipitate with Fehling's solution, the precipitin test being, as a rule, roughly parallel with the purity of the gum.

We must also leave open for the present the question of whether or not the yeast gum contains nitrogen or phosphorus in its molecule. The earlier workers state that it is nitrogen-free, but give no account

of how this was determined, and from the description of their method of preparation, certainly nothing approaching the efforts made in purifying our autoclave preparation were carried out by them.

The fact that the autoclave preparation fractions show a somewhat higher titer than the hot sodium hydroxide fractions is without significance, since a better serum was available for the latter part of the work. The difference in nitrogen content of these two purified preparations, 0.19 per cent in one and 0.56 per cent in the other, would seem to indicate that an impurity is still present in the autoclave preparation at least.

Attempts to Immunize Rabbits with Yeast Gum.

Previous attempts by Zinsser to immunize with bacterial residue antigen, and by Heidelberger and Avery with their pneumococcoccus specific substance, have been uniformly negative. We attempted in several different ways to bring about antibody production by injection of yeast gum, all with completely negative results.

In one experiment a rabbit was given intravenous doses averaging 50 mg. of dry gum, dissolved in saline at 5 day intervals for over 2 months. On several bleedings after the period of injections it showed no precipitins whatever.

In a second experiment, it was determined that the gum disappeared rather quickly from the circulation when given intravenously, but when administered intraperitoneally in doses of 0.25 gm., it appeared promptly in the blood, and was present for several hours after the injection. The rabbit was therefore given 0.35 gm. of the gum intraperitoneally every 12 hours for a week. The serum of the rabbit at all times during the week, and for about a week afterward, gave a ring test when diluted slightly with saline and layered upon yeast immune serum. But at no time, up to 2 weeks after the disappearance of the substance from the serum, could any evidence of precipitin production be found. The animal was then given a single intraperitoneal injection of 0.5 gm. compressed yeast killed at 60° for ½ hour, and a week later its serum showed a titer of about 1:10,000 with the gum. This merely served as a control on the rabbit to show that it could produce antibodies to yeast.

The third experiment was based upon Landsteiner and Simms' (9) experiments in connection with heterogenetic antibody production, and the so called "haptenes." They found that by mixing an alcohol extract of horse kidneys with dilute human serum or pig serum and injecting rabbits, antibodies to guinea pig cells were formed as was the case when suspensions of horse kidney were injected. They suggested that possibly the bacterial residue antigens were com-

parable to these haptenes, and served to carry specificity, but did not become antigenic until combined with protein.

Using Landsteiner and Simms' method we prepared, therefore, two solutions, one containing 12 cc. of 5 per cent yeast gum and 230 cc. of saline, the other, 12 cc. of 5 per cent gum and 230 cc. of a 1:8 dilution of horse serum. 0.25 per cent of phenol was added to each. Two series of three rabbits each were injected intravenously with 5 cc. of the solutions at 7 day intervals, until seven injections had been given. Bleedings on the 7th and 14th days after the last injection showed no trace of precipitin against yeast gum, although the three rabbits receiving the serum-gum mixture all showed high titer precipitin with horse serum.

These experiments on immunization, like earlier ones with similar material from other sources, are uniformly negative, but because of the considerable quantity injected into some of the animals, they make it highly improbable that the nitrogen in the gum is in the form of protein.

CONCLUSIONS.

Residue antigen recognizable by the precipitin test can be prepared from yeast as from bacteria.

The active material appears to be identical with a complex carbohydrate, the "yeast gum" of Salkowski.

In the purest form of it obtained small amounts of both nitrogen and phosphorus are still present, either as impurities or as part of the molecule.

The yeast gum is not antigenic in the sense of producing antibodies.

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PERSISTENCE OF INSPIRED BACTERIA IN THE LUNGS OF ALCOHOLIZED MICE.

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In a preceding paper (1) on the presence of bacteria in the lungs of mice following inhalation, the rate of disappearance of the inhaled bacteria was shown to depend upon the variety of the microorganism used. Pneumococci are disposed of within a few hours and do not give rise to a general infection. Hemolytic streptococci, on the other hand, persist in the lungs for a much longer period of time and generalized septicemia frequently occurs. In the present study, an attempt has been made to determine the conditions under which pneumococci which have reached the lung may induce a local or general reaction. As mice rarely succumb with a generalized infection following inhalation of pneumococci, it seemed desirable to see what effect alcoholic intoxication would have on the penetration of inspired bacteria.

There is a general belief that alcohol exerts a harmful influence on the natural defences of the body. The fact that alcoholic intoxication increases the susceptibility of animals to a number of infections is already recognized. Abbott (2) found that the normal resistance of rabbits is markedly diminished when alcohol is administered daily to the stage of acute intoxication. Rubin (3) states that intoxicated rabbits succumb more readily to pneumococcus and streptococcus infection and that the leucocytes in the peritoneal exudate of alcohol-intoxicated rabbits phagocyte fewer carmine granules than do leucocytes from normal rabbits. According to Friedenwald (4), except for a slight increase in the eosinophils, there is little difference between the blood of normal and the blood of intoxicated rabbits. Paulson (5) states that half the minimal lethal dose of acetonitrile is sufficient to kill guinea pigs which have received moderate quantities of alcohol.

In the present study, a comparison has been made in normal and intoxicated mice of the time of disappearance from the lungs of inspired pneumococci, hemolytic streptococci, and *Bacillus influenzæ*,

and the relative occurrence of generalized infection in the two groups of animals following the inhalation of pneumococci.

Method.

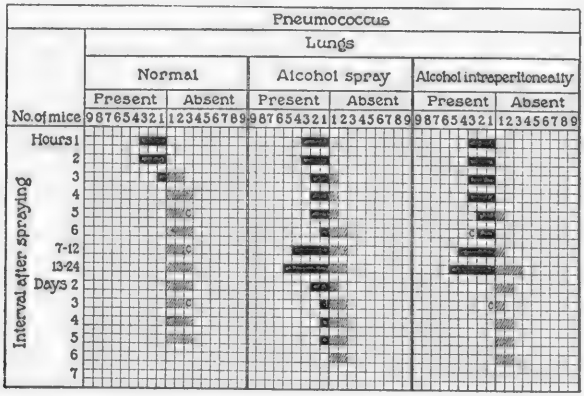
Mice were used as test animals. They were allowed to inhale an atmosphere in which a fine bacterial mist had been produced by spraying a culture of the organism to be studied. The organisms used were Pneumococcus Type I, *Streptococcus hæmolyticus*, and *B. influenzæ*. The animals were placed in the spraying chamber previously described (1), and were sprayed with 50 cc. of broth culture of the test organism. As a rule, the mice were removed from the spray box after an exposure of 1 hour. At various intervals following spraying, individual mice were killed. In order to prevent the possibility of aspiration of bacteria from the upper to the lower respiratory tract at the time of death, the mice were killed instantaneously by clamping the trachea and spinal cord with sponge forceps. The mice were then immersed in a solution of lysol and autopsied with sterile instruments. As a routine procedure, small pieces of the lung and a few drops of the heart's blood were separately cultured in plain broth. All positive cultures were plated on blood agar for further identification.

The mice were intoxicated either by inhalation of an alcoholic spray or by intraperitoneal injection of 1.5 cc. of 10 per cent alcohol in salt solution. In the first procedure, the mice were allowed to remain in the spray box 30 to 45 minutes after spraying with alcohol by which time they were under the influence of it. This method, however, gave irregular results. Some of the mice were relatively unaffected while others died later from the acute alcohol poisoning. For this reason the second procedure, in which the alcohol was administered intraperitoneally, was adopted. By the injection method, the average 20 gm. mouse becomes completely stupefied within 3 minutes and remains so for from 3 to 4 hours. The tolerance of individual mice to alcohol varies greatly. As a rule, however, mice show little evidence of intoxication on the day following the administration of alcohol.

In Text-figs. 1 to 4 each black square indicates one mouse from the lungs or heart's blood of which the sprayed bacteria were recovered. The cross-hatched squares represent those mice from which the test organisms were not recovered. In a few instances, especially in the cultures from the lungs, the identification of the sprayed organism was not possible because of contamination by other bacteria. All lung or blood cultures so contaminated are indicated in the text-figures by the letter *c*.

EXPERIMENTAL.

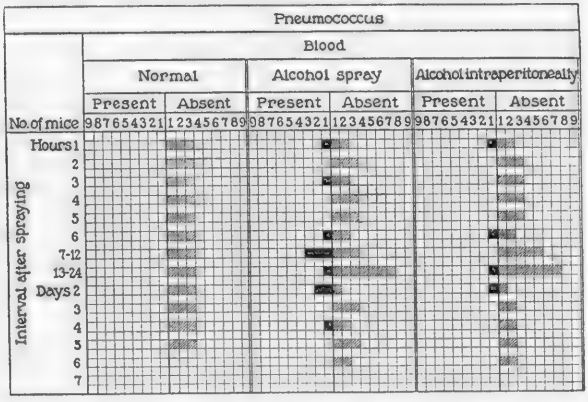
In Text-fig. 1 are shown the number of times pneumococci were recovered from the lungs of mice which were killed at stated intervals following spraying pneumococci into the air breathed by the animals. It is seen that while pneumococci were almost invariably recovered from the lungs within 3 hours following spraying, after 4 hours they could no longer be demonstrated by cultural methods. In the alcohol-treated mice, however, the sprayed bacteria were recovered from the lungs quite regularly during the first 24 hours, and in one instance at least were demonstrated in the tissue after 5 days. From this



TEXT-FIG. 1. Recovery of pneumococci from the lungs of normal and intoxicated mice following spraying. Each black square represents one mouse from whose lungs the sprayed bacteria were recovered. The cross-hatched squares represent those mice from whose lungs the sprayed bacteria were not recovered.

experiment, it is clear that pneumococci may be recovered from the lungs of normal mice only for a short period following exposure to an atmosphere containing pneumococci in finely divided droplets. On the other hand, if mice are intoxicated with alcohol, the normal mechanism for the disposal of the inspired bacteria is interfered with and in consequence of this the period of their persistence in the lungs is much prolonged.

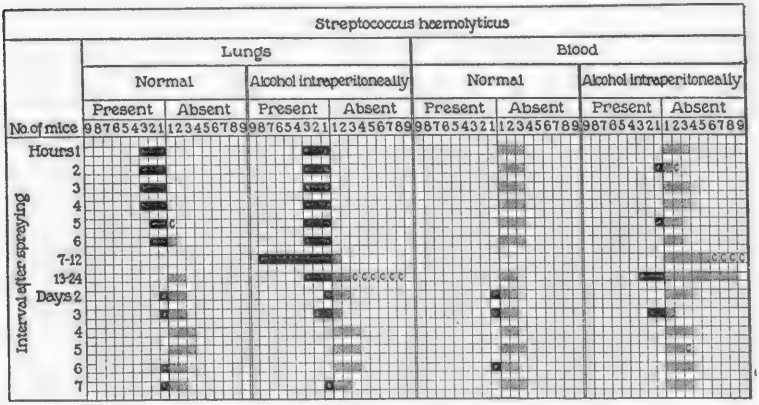
This inhalation experiment shows that pneumococci which are implanted on the lung tissue remain viable for several days in the lungs of intoxicated mice. In order to determine whether the inspired bacteria were merely implanted on the surface of the bronchi, or if invasion of the lungs had actually occurred, cultures were made of the heart's blood in each instance. The results of the cultures of the heart's blood of the 36 normal and 87 intoxicated mice is shown in Text-fig. 2. The blood cultures of the 36 normal mice remained sterile. Of the 87 mice treated with alcohol previous to their exposure, fourteen, or 16 per cent, showed the presence of the type



TEXT-FIG. 2. Recovery of pneumococci from the blood of normal and intoxicated mice following spraying. Each black square represents one mouse from whose blood the sprayed bacteria were recovered. The cross-hatched squares represent those mice from whose blood the sprayed bacteria were not recovered.

organisms in their blood. It is surprising to note that in at least two instances the blood was invaded within 1 hour after spraying. This experiment demonstrates that when normal mice are allowed to breathe an atmosphere in which pneumococci have been suspended by spraying, the inspired bacteria rarely invade the tissue in sufficient numbers to cause septicemia. If, however, mice are intoxicated previous to the inhalation of pneumococci, the inhaled bacteria are able, in a considerable number of instances, to invade the host in sufficient numbers to cause septicemia.

In order to determine the influence of alcohol upon the occurrence of infection, a number of experiments were performed, in which the technique was similar to that already described. In these experiments, however, the mice were not killed, but were kept under observation for a period of at least 14 days to contrast the mortality from infection in the two groups of animals. Of the 54 normal mice only two succumbed during this period, and in both instances pneumococci were recovered from the heart's blood. 50 of the 102 intoxicated mice died. From the blood of 45, or 44 per cent, pneumococci were recovered, while in five the blood remained sterile. From these experiments it is seen that alcohol



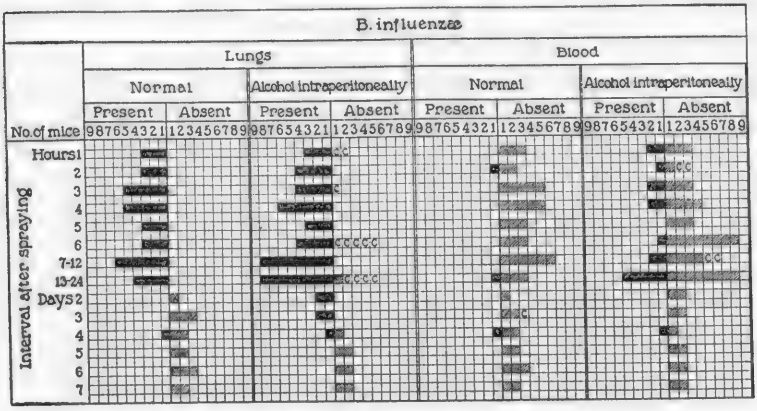
TEXT-FIG. 3. Recovery of *Streptococcus hæmolyticus* from the lungs and blood of normal and intoxicated mice following spraying. Each black square represents one mouse from whose lungs or blood the sprayed bacteria were recovered. The cross-hatched squares represent those mice from whose lungs or blood the sprayed bacteria were not recovered.

intoxication so alters the natural defensive mechanism of the body that inspired pneumococci may invade in sufficient numbers to cause death by septicemia.

In order to determine how alcohol would affect the persistence of other bacteria, inhalation experiments were performed with *Streptococcus hæmolyticus* and *Bacillus influenzæ*.

The results of spraying 38 normal and 57 intoxicated mice with *Streptococcus hæmolyticus* are seen in Text-fig. 3. Hemolytic streptococci were recovered from the lungs of sixteen of the eighteen normal

mice killed within 6 hours after spraying but only in four, or 20 per cent, of the twenty mice killed from 1 to 7 days following exposure. In the case of intoxicated mice, hemolytic streptococci were cultured from the lungs of twenty-six, or 96 per cent, of the twenty-seven mice killed within 12 hours of spraying and from seven of the thirty mice killed from 1 to 7 days after spraying. It is remarkable that in two instances this organism was recovered from the lungs on the 7th day after spraying. There is little difference in the rapidity of disappearance of this organism from the lungs of normal and intoxicated mice. The hemolytic streptococcus, however, was demonstrated in the blood of only three, or 7 per cent, of the 38 normal



TEXT-FIG. 4. Recovery of *B. influenzæ* from the lungs and blood of normal and intoxicated mice following spraying. Each black square represents one mouse from whose lungs or blood the sprayed bacteria were recovered. The cross-hatched squares represent those mice from whose lungs or blood the sprayed bacteria were not recovered.

mice and did not appear in the blood until the 2nd day following spraying. In the 57 intoxicated mice, however, this organism was recovered from the blood in seven instances (12 per cent) and appeared in the blood as early as 2 hours after spraying.

In Text-fig. 4 are seen the results of spraying 46 normal and 66 intoxicated mice with *Bacillus influenzæ*. This organism was recovered from the lungs of all the 32 normal mice killed within 24

hours of spraying and was present only once in the lungs of the remaining fourteen mice. In the intoxicated mice, *Bacillus influenzæ* was likewise recovered from the lungs of all animals killed within 12 hours. The blood was invaded in only three, or six per cent, of the normal mice, while septicemia occurred in sixteen, or 24 per cent, of the intoxicated animals. Not only was the incidence of septicemia higher in the intoxicated mice, but the organism persisted in the lungs for a longer period.

The results as regards the persistence in the lungs and invasion of the blood stream are not so striking with hemolytic streptococci and *Bacillus influenzæ* as in the case of pneumococci. The organisms first mentioned are not so rapidly removed from the lungs of normal mice and occasionally invade the blood even in normal animals. However, even with these organisms there is a greater tendency to blood invasion in the intoxicated animals, as evidenced by the increased number of positive blood cultures.

DISCUSSION.

The results of these experiments support the previous observations that in mice exposed to a dense spray containing bacteria in suspension, the inhaled organisms penetrate to the smaller bronchi. The further history of the bacteria which have been implanted in the lungs by inhalation depends not only upon the kind, number, and invasive quality of the bacteria, but also upon the normal defensive mechanism of the host. The difference in the invasive powers of the bacteria studied, and the variations in the number of bacteria gaining entrance to the respiratory tract following spraying are not subject to experimental control. The varying results of the present experiments are in part dependent upon these facts. The ability of the host to resist infection may, however, be somewhat depressed by experimental procedures. In the present instance the depressive influence of alcohol was successfully used to alter the normal defensive mechanism of the body against bacterial invasion.

Pneumococcus is an organism which is highly virulent for normal mice when given by *intraperitoneal* or subcutaneous inoculation. When this organism, however, is implanted on the respiratory mucosa

of normal mice by inhalation, it disappears from the lungs within a few hours and rarely gives rise to infection. If the normal mechanism of disposal, however, is interfered with by the administration of alcohol, inspired pneumococci persist in the lungs of mice for a longer time and frequently invade the blood. Thus, in the inhalation experiments less than 3 per cent of the normal mice died of pneumococcus septicemia, while over 40 per cent of intoxicated animals succumbed to fatal blood infection. Hemolytic streptococci and *Bacillus influenzæ* persist in the lungs of normal mice for a much longer time, and the difference between intoxicated and normal mice is not so distinct. However, in intoxicated mice the blood is apparently not only more rapidly, but also more frequently, invaded even by these organisms.

These experiments throw no light on whether the inspired bacteria are killed *in situ*, are taken up by leucocytes, or are carried to other parts of the body. Neither do they show in what manner the alcohol exerts its harmful effects, or whether alcohol intoxication causes local changes in the permeability of the respiratory mucosa, or acts adversely on the systemic resistance. The experiments therefore fail to shed any light on the exact mode of natural infection of the lungs with pneumococci. But they do show that alcohol delays the removal of inspired bacteria from the lungs and favors the invasion of the blood stream.

CONCLUSIONS.

1. When mice are exposed to an atmosphere containing certain bacteria in the form of a fine mist, the bacteria may be recovered from the deeper respiratory passages.

2. Pneumococci which have reached the lungs of normal mice as a result of this procedure usually disappear within a few hours and give rise to no generalized infection. In mice intoxicated with alcohol, on the other hand, pneumococci persist in the lungs for a longer period and fatal speticemia frequently follows.

3. Hemolytic streptococci and *Bacillus influenzæ* generally persist in the lungs for about 24 hours. In intoxicated mice these organisms do not disappear so rapidly from the lungs and generalized infection is much more frequent.

4. The experiments yield no evidence as to how alcoholic intoxication renders the lungs more permeable to inspired bacteria.

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EFFECT OF FATTY ACIDS ON THE RESISTANCE OF MICE TO TRANSPLANTED CANCER.*

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Soon after the discovery of the fact that resistance against transplanted cancer can be produced in animals by preliminary injections of homologous living cells,[1] it was found that neither serum nor plasma has such an immunizing action, nor are killed cells potent in bringing it about.[2] These facts have lead to a general conclusion that "some subtle product of metabolism of the living cell" is essential in producing immunity, and that cell-free substances are ineffective as "antigens."[3] Nevertheless, I have been able to show recently that a state of increased resistance, in every way comparable to that induced by living cells, can be engendered in mice by the injection of a suitable quantity of olive oil.[4] This work had for its starting point the observation that a lymphoid cell reaction follows the injection of fatty oil,[5]—a reaction essentially similar to that which ensues after an injection of homologous living cells.[6,7] Pursuing the investigation, I found it

* This investigation was carried out by means of funds from the Rutherford Donation.

[1] Bashford, E. F., *Brit. Med. J.*, 1906, ii, 209. Bashford, E. F., Murray, J. A., and Cramer, W., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 180. Schöne, G., Münch. med. Woch., 1906, liii, 2517. Ehrlich, P., *Arb. k. Inst. exp. Therap. Frankf. a. M.*, 1906, i, 75.

[2] Bashford, E. F., Murray, J. A., and Cramer, W., Third Scientific Report, Imperial Cancer Research Fund, London, 1908, 333, 367. Lewin, C., *Z. Krebsforsch.*, 1907–08, vi, 308. Haaland, M., *Proc. Roy. Soc. London, Series B*, 1909–10, lxxxii, 293.

[3] For the review of literature see Woglom, W. H., Studies in cancer and allied subjects. The study of experimental cancer, a review, New York, 1913, i.

[4] Nakahara, W., *J. Exp. Med.*, 1922, xxxv, 493.

[5] Bergel, S., *Berl. klin. Woch.*, 1919, lvi, 915.

[6] da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1.

[7] Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

possible, by a judicious use of olive oil, to induce a general stimulation of lymphoid tissue such as occurs in mice which have been rendered resistant to cancer inoculation by injections of living cells. Shortly after the appearance of my communication, Bierich[8] reported that an increased resistance to cancer transplantation could be induced by means of an unsaturated fatty acid, the name of which he failed to give.

In experiments to be reported in the present paper, I have attempted to throw a further light on the effect of fatty acids on the resistance to transplanted cancer by testing the influence of the following substances: sodium oleate, sodium palmitate, sodium stearate, oleic acid, linolic acid, and linolenic acid.

Experimental Methods.

The soaps were made up in 1 per cent solutions with distilled water and were administered in the form of a single intraperitoneal injection. Fatty acids were similarly injected in 1 per cent emulsion. In general these latter were emulsified with distilled water to which an appropriate amount of sodium hydroxide was added so as to make the emulsion fairly stable. In the case of oleic acid, the emulsion was sometimes prepared from a sodium oleate solution by adding a sufficient amount of hydrochloric acid. The oleic acid emulsion thus obtained was found not to differ in its action from the one prepared directly from oleic acid.

In testing the resistance to transplanted cancer of mice thus injected, an interval of 10 days was allowed to elapse between the injection and the implantation of cancer (Bashford Adenocarcinoma No. 63.) In each experiment, as control, a suitable number of untreated normal mice of the same breed were implanted at the same time with the same tumor. The implantation was made through a hollow needle, according to the established technique, into the subcutaneous tissue of the left groin, and the rate of growth of tumors was charted weekly thereafter. All the mice used were young adults of about the same size, weighing from 17 to 20 gm. each.

[8] Bierich, R., Leeuwenhoek-Vereeniging, 1922, i, 14.

Immunizing Action of Soaps.

My first task was to compare the action of unsaturated and saturated fatty acids. This I have attempted to do by testing sodium salts of oleic acid (unsaturated), palmitic acid, and stearic acid (saturated).

Sodium Oleate. Experiments 1 to 5.—Sodium oleate (Merck) was injected in 2 to 6 mg. amounts (0.2 to 0.6 cc. of 1 per cent solution). The incidence of resistance to the transplanted cancer exhibited by the treated mice as contrasted with that of the normal controls is shown in Table I.

TABLE I.

Effect of Sodium Oleate on Resistance to Implanted Cancer. Experiments 1 to 5.

Experiment No.	Amount of sodium oleate.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	mg.				
1	2	7	2 (28.5 per cent).	9	1 (11.1 per cent).
2	4	12	5 (41.6 " ").	9	1 (11.1 " ").
3	6	8	5 (62.5 " ").	9	2 (22.2 " ").
4	6	12	4 (33.3 " ").	10	0 (0.0 " ").
5	6	16	9 (56.2 " ").	10	1 (10.0 " ").
Average for 6 mg.....		36	18 (50.0 per cent).	29	3 (10.3 per cent).

The above results demonstrate that injections of sodium oleate in amounts of 4 to 6 mg. each produce a material increase in the resistance of mice to subsequent cancer transplantation. All the mice were in good physical condition, though an amount of sodium oleate larger than that used in these experiments, if given in a single injection, produced ill effects on their general health.

Sodium Palmitate. Experiments 6 to 11.—Sodium palmitate (Merck) was injected into mice in the form of 1 per cent solution in amounts varying from 2 to 12 mg. Cancer implants were made 10 days after the soap injection. The results of six experiments were as follows (Table II):

TABLE II.

Effect of Sodium Palmitate on Resistance to Implanted Cancer. Experiments 6 to 11.

Experiment No.	Amount of sodium palmitate.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	mg.				
6	2	8	0 (0.0 per cent).	9	1 (11.1 per cent).
7	4	10	2 (20.0 " ").	9	1 (11.1 " ").
8	6	10	3 (30.0 " ").	9	2 (22.2 " ").
9	8	10	2 (20.0 " ").	9	2 (22.2 " ").
10	10	9	1 (11.1 " ").	8	2 (25.0 " ").
11	12	10	2 (20.0 " ").	8	2 (25.0 " ").
Average.....		57	10 (17.5 per cent).	52	10 (19.2 per cent).

It is evident that sodium palmitate, unlike sodium oleate, does not exert an immunizing action on mice against transplanted cancer, at least when given in the amounts employed in the above experiments.

Sodium Stearate. Experiments 12 to 17.—Various amounts of sodium stearate (Merck) were injected into mice in the form of 1 per cent solution and the resistance of the animals subsequently tested. Table III gives the results.

TABLE III.

Effect of Sodium Stearate on Resistance to Implanted Cancer. Experiments 12 to 17.

Experiment No.	Amount of sodium stearate.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	mg.				
12	2	10	2 (20.0 per cent).	7	1 (14.2 per cent).
13	4	7	1 (14.2 " ").	7	1 (14.2 " ").
14	6	10	2 (20.0 " ").	11	2 (18.1 " ").
15	8	10	1 (10.0 " ").	11	2 (18.1 " ").
16	10	8	2 (25.0 " ").	9	2 (22.2 " ").
17	12	6	1 (16.6 " ").	9	2 (22.2 " ").
Average.....		51	9 (17.6 per cent).	54	10 (18.5 per cent).

As in the case of sodium palmitate, no perceptible difference can be noted in the incidence of resistance in mice treated with sodium stearate and that of untreated controls.

Immunizing Action of Unsaturated Fatty Acids.

The foregoing experiments show that sodium oleate has a marked immunizing action on mice against transplanted cancer, and that sodium palmitate and sodium stearate are wholly without such effect. These observations, coupled with our previous results with olive oil[4] lead one to question whether oleic acid may not be responsible for the positive findings. In order to test this point and to determine, furthermore, the influence of unsaturation in the reaction, we have tested three unsaturated fatty acids, representatives of three different chemical series; *viz*, oleic acid, linolic acid, and linolenic acid.

Oleic Acid. Experiments 18 to 23.—A 1 per cent emulsion of oleic acid was injected intraperitoneally into mice in doses varying from 0.4 to 0.8 cc. each (0.004 to 0.008 cc. of oleic acid). In Experiments 18, 20, and 23, the emulsion used was prepared from sodium oleate (Merck) in the manner already described. The emulsion used for other experiments was made directly from oleic acid (Eimer and Amend). The cancer implantation was carried out 10 days after the injection of the emulsion. The results are summarized in Table IV.

TABLE IV.

Effect of Oleic Acid on Resistance to Implanted Cancer. Experiments 18 to 23.

Experiment No.	Amount of oleic acid.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	cc.				
18	0.004	9	3 (33.3 per cent).	6	1 (16.6 per cent).
19	0.004	10	8 (80.0 " ").	10	2 (20.0 " ").
20	0.005	10	7 (70.0 " ").	12	2 (16.6 " ").
21	0.006	8	6 (75.0 " ").	6	1 (16.6 " ").
22	0.006	11	3 (45.4 " ").	10	0 (0.0 " ").
23	0.008	13	8 (61.5 " ").	11	4 (36.3 " ").
Average.....		61	37 (60.6 per cent).	55	10 (18.1 per cent).

The results of these experiments provide conclusive evidence that oleic acid is highly potent in increasing the resistance of mice to transplanted cancer. It is noteworthy that this action of oleic acid was obtained with all of the various amounts employed.

Linolic Acid. Experiments 24 to 30.—A 1 per cent linolic acid (Kahlbaum) emulsion was injected into mice in amounts varying from 0.2 to 0.8 cc. each (0.002 to 0.008 cc. of linolic acid). The resistance to transplanted cancer shown by the treated mice as contrasted to that of untreated controls is recorded in Table V.

TABLE V.

Effect of Linolic Acid on Resistance to Implanted Cancer. Experiments 24 to 30.

Experiment No.	Amount of linolic acid.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	cc.				
24	0.002	10	3 (30.0 per cent).	10	4 (40.0 per cent).
25	0.004	10	5 (50.0 per cent).	10	2 (20.0 per cent).
26	0.004	10	5 (50.0 " ").	10	2 (20.0 " ").
27	0.005	10	6 (60.0 " ").	12	2 (16.6 " ").
28	0.006	10	6 (60.0 " ").	10	2 (20.0 " ").
29	0.006	10	5 (50.0 " ").	10	2 (20.0 " ").
Average for 0.004 to 0.006 cc.....		50	27 (54.0 per cent).	52	10 (19.2 per cent).
30	0.008	7	3 (42.8 per cent).	11	3 (27.2 per cent).

It is evident from the above figures that linolic acid is capable of producing increased resistance to cancer inoculation, and that the optimum doses are from 0.004 to 0.006 cc. each.

Linolenic Acid. Experiments 31 to 37.—Linolenic acid (Kahlbaum) was injected into mice in the form of a 1 per cent emulsion in varying amounts. The outcome of cancer implantation 10 days after the linolenic acid injection was as follows (Table VI):

TABLE VI.

Effect of Linolenic Acid on Resistance to Implanted Cancer. Experiments 31 to 37.

Experiment No.	Amount of linolenic acid.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	cc.				
31	0.002	10	5 (50.0 per cent).	10	4 (40.0 per cent).
32	0.004	10	10 (100.0 per cent).	10	4 (40.0 per cent).
33	0.004	10	6 (60.0 " ").	10	2 (20.0 " ").
34	0.005	10	4 (40.0 " ").	12	2 (16.6 " ").
Average for 0.004 to 0.005 cc.....		30	20 (66.6 per cent).	32	8 (25.0 per cent).
35	0.006	10	2 (20.0 per cent).	11	4 (36.3 per cent).
36	0.006	6	1 (16.6 " ").	10	2 (20.0 " ").
37	0.008	9	3 (33.3 " ").	11	4 (36.3 " ").

The above result warrants the conclusion that linolenic acid shares, with other unsaturated fatty acids already tested, the immunizing action against subsequent cancer inoculation. The range of optimum doses here is apparently somewhat less than in the case of linolic acid and it is decidedly less than that of oleic acid.

Influence upon Previously Implanted Cancer Grafts.

With the immunizing action of unsaturated fatty acids against subsequent cancer inoculation established, attention was next turned to the possible effect of these substances on the growth of cancer grafts already implanted.

Sodium Oleate. Experiments 38 to 41.—Mice were implanted with Bashford adenocarcinoma in the usual manner. They were divided into two groups, and the mice in one group were injected with 0.5 cc. of 1 per cent solution of sodium oleate 2 or 48 hours after the implantations. The other group remained untreated and served as control. The results of four similar experiments are summarized in Table VII.

TABLE VII.

Effect of Sodium Oleate on Cancer Grafts Previously Implanted. Experiments 38 to 41.

Experiment No.	Interval between tumor inoculation and the soap injection.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	hrs.				
38	2	15	7 (46.6 per cent).	10	3 (30.0 per cent).
39	2	7	3 (42.8 " ").	10	2 (20.0 " ").
40	48	20	3 (15.0 " ").	10	0 (0.0 " ").
41	48	8	1 (12.5 " ").	8	0 (0.0 " ").
Average.....		50	14 (28.0 per cent).	38	5 (13.1 per cent).

Oleic Acid. Experiments 42 to 44.—Similar experiments were carried out to test the effect of oleic acid, injected in 0.5 cc. of 1 per cent emulsion following the cancer implantation. Table VIII summarizes the result.

TABLE VIII.

Effect of Oleic Acid on Cancer Grafts Previously Implanted. Experiments 42 to 44.

Experiment No.	Interval between tumor inoculation and oleic acid injection.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	hrs.				
42	2	15	6 (40.0 per cent).	10	3 (30.0 per cent).
43	48	7	2 (28.5 " ").	7	1 (14.2 " ").
44	48	9	4 (44.4 " ").	10	2 (20.0 " ").
Average.....		31	12 (38.7 per cent).	27	6 (22.2 per cent).

Linolic Acid. Experiments 45 to 47.—The effect of 0.5 cc. of 1 per cent emulsion of linolic acid was similarly tested in three experiments (Table IX).

TABLE IX.

Effect of Linolic Acid on Cancer Grafts Previously Implanted.
Experiments 45 to 47.

Experiment No.	Interval between tumor inoculation and linolic acid injection.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	hrs.				
45	2	10	3 (30.0 per cent).	10	2 (20.0 per cent).
46	48	9	3 (33.3 " ").	7	1 (14.2 " ").
47	48	18	4 (22.2 " ").	10	1 (10.0 " ").
Average.....		37	10 (27.0 per cent).	27	4 (14.8 per cent).

Linolenic Acid. Experiments 48 to 50.—In these experiments the effect of 0.5 cc. of 1 per cent emulsion of linolenic acid was tested as in the preceding experiments (Table X).

TABLE X.

Effect of Linolenic Acid on Cancer Grafts Previously Implanted.
Experiments 48 to 50.

Experiment No.	Interval between tumor inoculation and linolenic acid injection.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	hrs.				
48	2	10	2 (20.0 per cent).	10	2 (20.0 per cent).
49	48	8	3 (37.5 " ").	7	1 (14.2 " ").
50	48	20	2 (10.0 " ").	10	1 (10.0 " ").
Average.....		38	7 (18.4 per cent).	27	4 (14.8 per cent).

It is evident from the above results that no marked effect can be produced by any of these means on cancer grafts already implanted. There was a slightly lower per cent of tumor development in the treated mice than in the controls, but the difference by itself was too small to be considered significant. However, in treated mice, there were always two or three out of every eight or nine tumors that grew extremely slowly, or almost not at all. Upon microscopical examination these tumors were found to consist of small, discrete masses of

neoplastic cells surrounded by a dense layer of connective tissue with intense lymphocytic infiltration. No such retardation of growth occurred in tumors of the control series, all growing progressively. These findings suggest, rather than prove, that the fatty acids do exert some influence but that this is not adequate to modify markedly the development of a rapidly growing cancer such as the one used in these experiments. It may be recalled that all the hitherto discovered methods of inducing general resistance against subsequent cancer grafting fail when tested against grafts already *in situ*. This is probably because of the great rapidity with which most of the transplantable cancers grow, as also because the artificially induced state of resistance develops only after a latent period of several days.[1,4,9]

DISCUSSION.

The experiments reported in the present paper show conclusively that unsaturated fatty acids such as oleic, linolic, and linolenic acids have a distinct immunizing action against transplanted cancer in mice, and that this action is apparently not shared by saturated fatty acids. Of the unsaturated fatty acids used, oleic acid shows the widest range in the amounts capable of producing the desired effect, while the range with the other acids is more limited. There is no apparent difference in the activity of these unsaturated fatty acids in the optimum amounts. Whether the immunity-producing quality of fats can be estimated on the basis of their iodine values remains to be determined. In an unpublished observation it was noted that cocoanut oil which has an extremely low iodine value produces no resistance to cancer, and at the same time that cod liver oil, in spite of a very high iodine value, is equally impotent in this regard. However, it must be remembered that in dealing with such complex mixtures as these fats, factors other than fatty acids must be taken into consideration in interpreting biological effects.

A problem of broad interest suggested by these results is that of the possible part played by unsaturated fatty acids in cancer immunity induced by means other than injection of them.[9] In addition to the

[9] Woglom, W. H., *J. Exp. Med.*, 1912, xvi, 629. Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423.

older method of living tissue injection,[1] it has been possible to induce immunity by exposing mice to suitable doses of x-radiation[10, 11] or to intense dry heat.[12] It seems not unreasonable to suspect that these varied treatments may be at one in producing disturbances that involve some changes in the unsaturated fatty acid.

The possibility that the cancer immunity induced by unsaturated fatty acids may be consequent on increased activity of the lymphoid tissue deserves consideration. The close relationship existing between fat metabolism and the lymphoid tissue,[13] under both physiological and pathological conditions, and the clearly demonstrated association of lymphoid stimulation with cancer immunity[7, 11, 12, 14] are in favor of this view. As a matter of fact, we have found that injections of sodium oleate in amounts sufficient to produce immunity bring about a marked increase in the number of karyokinetic figures in the lymphoid tissue, indicating an increased proliferative activity of this tissue.

SUMMARY.

Sodium oleate, oleic acid, linolic acid, and linolenic acid injected into mice in suitable amounts induce a material increase in the resistance against subsequent transplantation of cancer grafts, although they fail to exert so marked an influence on cancer grafts already in place.

Sodium palmitate and sodium stearate, on the other hand, do not produce immunity, at least in the amounts employed in the present study.

[10] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692.

[11] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

[12] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 25.

[13] Schäfer, E. A., *Internat. Monatschr. Anat. u. Histol.*, 1885, ii, 6. Bergel, S., *Ergebn. inn. Med. u. Kinderheilk.*, 1921, xx, 36.

[14] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204. Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.

A CARDIOVALVULOTOME.

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Plates 14 and 15.

(Received for publication, May 1, 1924.)

The instrument and the operative procedure described here were developed with the purpose of establishing a surgical method applicable to the treatment of mitral stenosis.

With the same intention investigators during the past 20 years have developed methods for creating valvular insufficiency in animals. Cushing and Branch (1), Haecker (2), and Schepelmann (3) used small knives and hooks on long handles which were thrust into the ventricles and manipulated to slit or tear out valve cusps. Tuffier and Carrel (4) developed an extracardiac method consisting of suturing a piece of vein over the region of the pulmonic orifice, then dividing the ring with fine scissors slipped beneath this covering, thus allowing the support of the valve cusps to bulge outwards and produce an insufficiency. Recently Allen and Graham (5) have used an endoscope carrying a small knife with which the valves can also be divided.

Application of such methods to cases of mitral stenosis in man is limited to three attempts. The first was by Tuffier (6), who in 1912 attempted to stretch the stenosed aortic valve in a young man by introducing a finger into the stenosed ring through the invaginated wall of the aorta. The second was by Doyen (7), who unsuccessfully attempted to section the stenosed mitral valve, death being due to a lesion in the interventricular septum. The third is the case reported by Cutler and Levine (8) in which an attempt was made to section the stenosed mitral valve with a knife inserted through the left ventricle.

When the condition of stenosis in cases of chronic valvular disease is considered, the degree of obstruction, the thickness of the valve, its fibrous and often calcareous condition, it is at once apparent that such a valve, if valve it may be called, cannot be sectioned satisfactorily by a force applied to a knife several inches from the cutting edge without the aid of counterpressure applied to the valve at the point where the section is to be performed. The toughness of such a valve is so marked in some cases that, indeed, even scissors with

delicate blades are unable to section the valve under direct vision. It seems, furthermore, that a slit-like cut in the markedly thickened valve, on account of the stiffness and the rigidity of the incised margin, may relieve the functional obstruction but little, and that the excision of a piece from such a valve with its removal from the blood stream is almost a necessity if the obstruction is to be relieved. It seems essential, therefore, that a powerful cutting device be employed which will actually excise a piece from the stenosed ring and remove the piece from the blood stream (Fig. 1). A valvulotome has been devised which may aid in the accomplishment of this aim.

In the development of the instrument two guiding principles were accepted from the beginning as a necessity; namely, the principle of cutting by counterpressure and the application of force in a line parallel to the axis of the instrument by telescoping the handle. This latter principle makes the instrument straight and helps greatly in localization when the instrument is in the heart and when the sense of position is so important. In utilizing the principle of counterpressure, the punch is entirely inadequate in cutting a calcareous valve, and to have one cutting edge revolve upon the other as it cuts spends too much force in friction and in overcoming inertia. The shear, however, affords the ideal mechanical advantage.

The valvulotome (Fig. 3) that has been devised consists of two, closed curved, shearing edges, which by compression of the handle approximate each other and accurately override so that the tissue lying between the cutting edges is excised and encased in the instrument. As the force applied to the handle is released, the cutting edges move apart by virtue of a spring concealed in the handle. The distal portion of the instrument is a bluntly pointed pyramid with a cutting edge on its proximal end, which, by telescoping the handle, approximates the opposing cutting edge of the shaft into which it accurately cups. The cutting edges are adjusted to employ the principle of the shear and this contributes to make the instrument a powerful cutting device.

The operative procedure as worked out in the laboratory is as follows:

The heart is exposed by the subperiosteal removal of the fifth rib, from the costochondral articulation laterally about 5 inches. A suture placed in the

apex provides for traction and handling of the heart. A region devoid of coronary vessels, as near the apex as possible, and away from the base of the anterior papillary muscle[1] is selected for the introduction of the valvulotome. Two sutures of silk taking a deep bite of muscle are placed in the area selected for the insertion of the instrument so that the four threads represent on the surface of the heart a square of 1 cm. These are the control sutures and they are of importance in the control of hemorrhage. A small scalpel placed between the control sutures so as to bisect the hypothetical square cuts the ventricular wall in a plane slightly oblique to the surface of the heart. If there is any bleeding, and experience has shown that there need be no bleeding, the control threads which have been crossed are made slightly taut. The valvulotome, after having been filled with salt solution to displace the contained air, is introduced through the incision into the cavity of the left ventricle and is then allowed to open. The cutting edges are introduced into the mitral ring, the position being determined accurately by the index finger of the left hand as it feels the end of the instrument through the invaginated wall of the left auricle. The mitral valve leaflets now lie between the cutting edges and, by telescoping the handle, parts of one or more of them are excised and encased in the instrument which is then removed from the heart. Slight traction upon the control sutures prevents any bleeding as the instrument is withdrawn and the wound is closed with one or two silk sutures (Fig. 4). The control sutures and the apex suture are removed and the heart is replaced in the pericardial cavity. The pericardium is sutured and before completely closing the pleura the lungs are expanded to expel the air from the chest and the wound is sutured in layers.

That this operative procedure is feasible upon dogs has been well demonstrated in the laboratory where in a series of thirty dogs twenty-four survived the operation, almost all of which were given a well marked mitral insufficiency (Fig. 2). Of the six operative fatalities two occurred immediately after making the incision at the base of the anterior papillary muscle preparatory to the insertion of the valvulotome, one was the result of hemorrhage, and three followed the creation of an excessive regurgitation due to the removal of too large a segment from the valve. The tolerance of the heart toward this procedure is evidenced by blood pressure tracings which show only

[1] The literature contains numerous references to the so called vital areas of the heart and they appear to lie in the region of and immediately adjacent to the auriculoventricular ring. To these vital areas our experience adds the region at the base of the anterior papillary muscle and this represents the only area near the apex of the left ventricle that should be avoided.

slight variations with the placing of sutures, the displacement of the heart, the insertion of the valvulotome, and the creation of an insufficiency. The feasibility of the operative procedure is shown further by an operation upon a human case in which a segment of the stenosed mitral valve was actually excised and removed from the heart.

SUMMARY.

1. A study of the fibrosed and often calcareous condition present in the mitral orifice in cases of chronic stenosing rheumatic disease demonstrated that a powerful instrument must be developed if we are to apply surgical relief in the treatment of the disease.

2. An instrument (cardiovalvulotome) is described which actually excises a segment from the mitral orifice and removes this from the blood stream.

3. The efficacy of the instrument has been tested by its use on the stenotic valves of diseased hearts removed at autopsy. Its feasibility has been shown by operations both upon animals and in a single human case.

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5. Allen, D. S., and Graham, E. A., Intracardiac surgery—a new method, *J. Am. Med. Assn.*, 1922, lxxix, 1028.
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7. Doyen, quoted by Tuffier (6).
8. Cutler, E. C., and Levine, S. A., Cardiotomy and valvulotomy for mitral stenosis. Experimental observations and clinical notes concerning an operated case with recovery, *Boston Med. and Surg. J.*, 1923, clxxxviii, 1023.

EXPLANATION OF PLATES.

PLATE 14.

FIG. 1. Photographs showing mitral stenotic valves. The insets are x-ray films showing calcium deposits in the valves; the insets to the left are of the valves before cutting, those to the right are of the excised fragments.

FIG. 2. Photograph showing mitral valve, Dog 1, 2 months after operation.

PLATE 15.

FIG. 3. Photographs and mechanical drawing of the valvulotome. The cutting edges show the shearing principle. By telescoping the handle the cutting edges override so that the distal portion cups into the shaft. The length of the instrument is 20 cm. and the inside diameter is 1 cm.[2]

FIG. 4. Drawing showing the operative procedure in the dog. The inset shows the distal portion of the valvulotome and the excised segments of the mitral valve.

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[2] The valvulotome is made by Codman and Shurtleff, Inc., of 120 Boylston Street, Boston, Mass., to whom it is a pleasure to express appreciation of their cooperation in the construction of the instrument.

FIG. 1.

FIG. 2.

(Beck and Cutler: Cardiovalvulotome.)

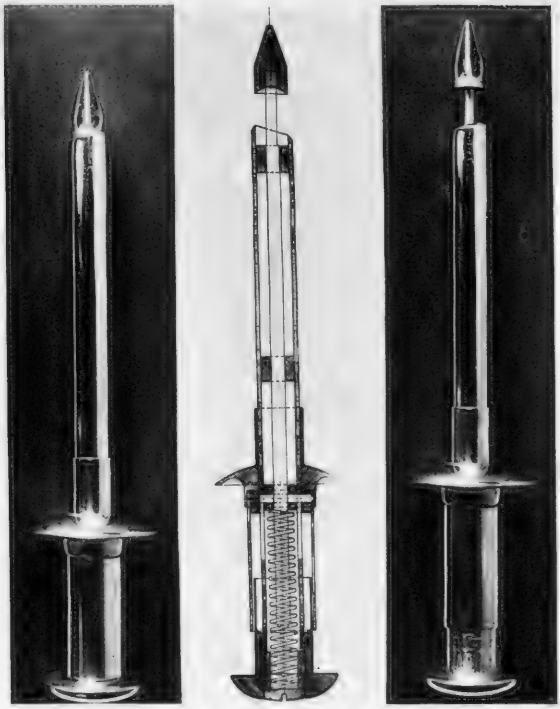

FIG. 3.

FIG. 4.

(Beck and Cutler: Cardiovalvulotome.)

OBSERVATIONS ON THE PRESENCE OF A TOXIC SUBSTANCE IN THE BLOOD AND URINE OF PATIENTS WITH SCARLET FEVER.

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Plate 16.

(Received for publication, April 25, 1924.)

Recent studies by Dochez,[1,2] Mair,[3] and Dick and Dick[4-6] have indicated that scarlet fever may be regarded as a local infection of the throat by a special type of *Streptococcus hæmolyticus* which is capable of producing a soluble toxic substance. It is supposed that this toxic substance is absorbed from the throat and causes the general manifestations of the disease. Whether this substance is a true exotoxin similar to diphtheria toxin has not as yet been established. It is supposed, furthermore, that immunity to scarlet fever, whether existing in a normal individual or acquired by an attack of the disease is antitoxic in nature and that the presence of immunity can be determined in an individual by finding out whether or not his serum blanches the rash in a patient with scarlet fever, or whether or not he reacts to an injection of the toxic substance found in filtrates of scarlatinal streptococcus cultures.

The experimental data on which the foregoing conception of scarlet fever is based may be briefly summarized.

[1] Dochez, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 184.
[2] Dochez, A. R., *J. Am. Med. Assn.*, 1924, lxxxii, 542.
[3] Mair, W., *Lancet*, 1923, ii, 1390.
[4] Dick, G. F., and Dick, G. H., *J. Am. Med. Assn.*, 1923, lxxxi, 1166.
[5] Dick, G. F., and Dick, G. H., *J. Am. Med. Assn.*, 1924, lxxxii, 265.
[6] Dick, G. F., and Dick, G. H., *J. Am. Med. Assn.*, 1924, lxxxii, 301.

Dochez and Bliss,[7] Bliss,[8,9] and Stevens and Dochez[10] have shown that strains of *Streptococcus hæmolyticus* from the throats of patients with scarlet fever are distinguishable immunologically from hemolytic streptococci from other sources and are themselves for the most part immunologically homologous. These observations have been independently substantiated by Tunnicliff[11,12] and by Gordon.[13]

Dochez[1,2] has shown, furthermore, that a disease resembling scarlet fever in many respects may be produced in guinea pigs by inoculation with scarlatinal streptococci by a method which establishes a local infection in the subcutaneous tissue of the animal. He has, in addition, produced an antiserum by immunization of a horse with living cultures of a scarlatinal streptococcus. This antiserum has been shown by Blake, Trask, and Lynch[14] to possess the same properties which human convalescent scarlet fever serum has been shown to possess; namely, the ability to blanch the scarlet fever rash at the site of an intracutaneous injection and to exert a favorable therapeutic effect on the disease. It is presumably antitoxic in character.

Dick and Dick[4,6] have succeeded in producing scarlet fever in human volunteers by inoculation of scarlatinal streptococci in the throats of the volunteers. They[5] have shown, furthermore, that bacteria-free filtrates of cultures of scarlatinal hemolytic streptococci contain a toxic substance which produces a local reaction when injected intracutaneously in persons supposedly susceptible to scarlet fever. This substance causes no reaction in persons who have had scarlet fever, or are supposedly immune to scarlet fever, or have been passively immunized by convalescent scarlet fever serum prior to the test with the toxic filtrate. The toxic action of the filtrate is neutralized by *in vitro* incubation with convalescent scarlet fever serum prior to injection in a susceptible person. Zingher[15] has repeated and substantiated these observations with the toxic filtrates of scarlatinal streptococcus cultures and has shown that the Dick test is, in many respects, analogous to the Schick test for susceptibility to diphtheria.

Mair,[3] in a study of the Schultz-Charlton[16] blanching phenomenon, has pointed out that the blanching of the scarlet fever rash by normal human serum or by convalescent scarlet fever serum may be more logically interpreted as an immunity

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[7] Dochez, A. R., and Bliss, W. P., *J. Am. Med. Assn.*, 1920, lxxiv, 1600.

[8] Bliss, W. P., *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 173.

[9] Bliss, W. P., *J. Exp. Med.*, 1922, xxxvi, 575.

[10] Stevens, F. A., and Dochez, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 39.

[11] Tunnicliff, R., *J. Am. Med. Assn.*, 1920, lxxiv, 1386.

[12] Tunnicliff, R., *J. Infect. Dis.*, 1922, xxxi, 373.

[13] Gordon, M. H., *Brit. Med. J.*, 1921, i, 632.

[14] Blake, F. G., Trask, J. D., Jr., and Lynch, J. F., *J. Am. Med. Assn.*, 1924, lxxxii, 712.

[15] Zingher, A., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 293.

[16] Schultz, W., and Charlton, W., *Z. Kinderheilk.*, 1918, xvii, 328.

reaction in which an antitoxin in the blanching serum neutralizes a toxin in the patient, than as a reaction due to some property of normal serum that is lost during the acute stage of scarlet fever as originally proposed by the discoverers of the phenomenon.

In order to provide further evidence in support of the foregoing conception of scarlet fever an effort has been made to determine whether a specific toxic substance could be demonstrated in the blood and urine of scarlet fever patients during the acute stage of the disease. The results of this study are presented in this paper.

Methods.

Samples of venous blood were collected from patients acutely ill with scarlet fever. After the blood had clotted, the serum was separated by centrifugation, pipetted off, and stored in the ice box in sterile bottles without preservative. Specimens of urine were filtered through Berkefeld filters and stored in a similar way. All specimens were tested for sterility before use. Tests for the presence of a toxic substance in the serum and urine samples were made by intracutaneous injections in two groups of human volunteers. Members of the first group were supposedly susceptible because it had been determined that their serums failed to blanch the rash of patients with scarlet fever. Members of the second group were supposedly insusceptible because their serums blanched the rash in patients with scarlet fever. Whenever a sample of serum or urine gave a positive local reaction, the specificity of the reaction was controlled by a neutralization test with a blanching serum. This was performed by intracutaneous injections in a known susceptible volunteer of two equal portions of the sample, one of which had been mixed with a human serum not possessing the property of blanching the rash of scarlet fever, the other of which had been mixed with a human serum possessing the property of blanching. The mixtures were incubated in a water bath at 37°C. for 30 minutes prior to injection. A few similar neutralization tests were also performed with normal horse serum in place of the non-blanching human serum and with Dochez's scarlatinal antistreptococcic serum in place of the human blanching serum. The volunteer test subjects were observed daily and the reactions obtained were studied with respect to size, duration, in-

tensity of the erythema, presence of induration, development of pigmentation, and the occurrence of desquamation.

Presence of a Toxic Substance in the Blood of Persons with Scarlet Fever.

For the preliminary tests specimens of serum from two severe cases of scarlet fever were selected.

I. Jan. 24, 1924. Blood was collected from M. R., a highly toxic case of scarlet fever, on the 3rd day of the disease. The serum, A, was separated from the clot and stored in the ice box. Feb. 16. The 21st day of convalescence, a second sample of blood was collected, the serum, B, separated and similarly stored.

Feb. 20, 9 a.m. 0.5 cc. of Serum A was injected intracutaneously on the flexor surface of the right forearm in each of three human volunteers, A. P., R. E., and M. B. Control injections of 0.5 cc. of Serum B were similarly made on the left forearms of A. P., R. E., and M. B. Blood serum from A. P. and R. E. had failed to blanch the rash of patients with scarlet fever, serum from M. B. had produced a well marked area of blanching. In the supposedly susceptible volunteers, A. P. and R. E., Serum A produced a marked local reaction in A. P. (Fig. 1), a definite but less severe local reaction in R. E., whereas the control Serum B produced no reaction (Fig. 2). In the supposedly insusceptible volunteer, M. B., neither Serum A nor Serum B produced any reaction. The character of the reactions is described in Table I.

II. 0.3 cc. of Serum A obtained from a severe case of scarlet fever, J. M., late on the 5th day of the disease was injected intracutaneously in each of five human volunteers, two of whom were supposedly susceptible since their serums had failed to blanch the rash of scarlet fever, and three of whom served as supposedly insusceptible controls since their serums had produced well marked positive blanching tests. Control injections of 0.3 cc. of Serum B obtained from the same patient on the 2nd day of convalescence were also made in each subject. The result was completely negative, no reaction occurring in any of the subjects with either serum.

In spite of the negative result obtained in the second test, the first one appeared to be sufficiently definite to warrant further investigation of a series of patients with scarlet fever, especially since it seemed possible that the positive reactions obtained in the first instance might be due to the fact that the blood was collected early on the 3rd day of the disease, while the negative result in the second instance might be due to the fact that the blood was collected late on the 5th day. At any rate, if the negative outcome of the second test

TABLE I.

Test for Toxic Substance in Serum of Scarlet Fever Patient M. R.

Volunteer test subjects.	Blanching power of serum.	Intracutaneous injections.	Character of local reaction at site of injections.				
		Feb. 20, 1924, 9 a.m.	Feb. 21, 10 a.m.	Feb. 22, 9 a.m.	Feb. 23, 10 a.m.	Feb. 24, 10 a.m.	Feb. 25, 10 a.m.
A. P.	−	Right arm, 0.5 cc. Serum A.*	Marked diffuse erythema 30 by 40 mm. Moderately indurated and tender.	Marked diffuse erythema 35 by 45 mm. Moderately indurated and tender.	Fading erythema 40 by 50 mm. Induration and tenderness gone.	Erythema faded. Moderate purplish brown pigmentation.	Slight remaining pigmentation.
		Left arm, 0.5 cc. Serum B† (control).	No reaction.	No reaction.	No reaction.	No reaction.	No reaction.
R. E.	−	Right arm, 0.5 cc. Serum A.	Moderate punctate erythema 30 by 45 mm. No induration or tenderness.	Moderate punctate erythema 30 by 55 mm.	Erythema faded. Blotchy purplish brown pigmentation 33 by 55 mm.	Faint brown pigmentation 33 by by 55 mm.	Completely faded.
		Left arm, 0.5 cc. Serum B (control).	No reaction.	No reaction.	No reaction.	No reaction.	No reaction.
M. B. (control).	+	Right arm, 0.5 cc. Serum A.	" "	" "	" "	" "	" "
		Left arm, 0.5 cc. Serum B.	" "	" "	" "	" "	" "

* Serum A was collected on the 3rd day of scarlet fever when the patient was extremely toxic.

† Serum B was collected on the 21st day of convalescence.

be disregarded for the moment, the result of the first would seem to show that a toxic substance capable of producing a well marked local reaction in the skin of supposedly susceptible individuals, but no reaction in supposedly insusceptible individuals, can be demonstrated in the blood serum early in the course of an instance of severe scarlet fever.

To provide further evidence on this point the following procedure was carried out.

III. Single specimens of serum were obtained from 17 cases of scarlet fever of varying degrees of severity; 2 were collected on the 2nd day of the disease, 9 on the 3rd day, 5 on the 4th day, and 1 on the 5th day.

Eight human volunteers were available for intracutaneous tests with the scarlet fever serums. Four of them, Group A, had not had scarlet fever, and samples of their serums when injected intracutaneously in patients with scarlet fever failed to blanch the rash. The other four, Group B, were used as control subjects. Two had had scarlet fever, two had not. Samples of serum from all four produced a well marked area of blanching when injected intracutaneously in patients with scarlet fever.

0.3 cc. of each scarlet fever serum was injected intracutaneously in one or more members of Group A and the resulting reactions were carefully observed. 0.3 cc. of each serum that gave a positive reaction in members of Group A was injected similarly in one or more members of Group B.

Six of the serums gave marked positive reactions in members of Group A, negative reactions in members of Group B. Five gave moderate positive reactions in the more susceptible members of Group A, negative reactions in members of Group B. Six gave no reaction in the more susceptible members of Group A and were therefore not tested in members of Group B.

The positive reactions varied from very marked reactions showing areas of bright diffuse erythema, 50 to 70 mm. in diameter, moderately indurated and tender, of 3 or 4 days duration, followed by pigmentation and desquamation, to mild reactions of moderate diffuse or blotchy erythema, 20 to 30 mm. in diameter, not indurated or tender, of 24 to 48 hours duration, followed by transient pigmentation and no desquamation.

The details are presented in Table II.

From the result of the foregoing test it is clear that there is a toxic substance present in the blood of many cases of scarlet fever in sufficient concentration to produce a clear-cut local reaction in the skin of normal individuals, provided the blood serum of those individuals gives a negative Schultz-Charlton rash extinction test in

TABLE II.

Test for Toxic Substance in Scarlet Fever Blood by Intracutaneous Injection in Man.

			Group A.				Group B.			
Volunteer test subjects			F.G.B.	E.E.N.	I.H.	E.McC.	J.D.T.	J.C.F.	R.A.G.	M.G.
History of scarlet fever			0	0	0	0	0	+	+	0
Blanching power of serum			0	0	0	0	+	+	+	+
Scarlet fever patients.			Reaction to intracutaneous injection of 0.3 cc. of scarlet fever serum.							
Case.	Clinical severity.	Day of disease.								
1. A. D.	Very severe, septic.	5	+++	+++	++	++	−	−	−	−
2. J. D.	Mild.	3	+++	++	++	++	−	−	−	−
3. R. M.A.	Moderately severe.	2	+++				−			
4. C. M.	Very toxic.	3	++	++	+	++	−	−	−	−
5. N. G.	Moderately severe.	3	++	++	+	+	−	−	−	−
6. A. C.	Very severe, septic.	4	++				−			
7. A. Z.	Severe, septic.	4	+	+	+	−	−	−	−	−
8. L. W.	"	4	+	+	−	−	−	−	−	
9. S. S.	Moderately severe.	3	+	+	−	−	−	−	−	−
10. H. C.	Mild.	3	+	+	−	−	−	−	−	
11. C. P.	Moderately toxic.	3	+	+	−	−	−	−	−	
12. P. N.	Mild.	3	−		−	−				
13. F. E.	"	3	−							
14. A. W.	"	4	−	−						
15. A. D.	"	3	−	−						
16. M. D.	Moderately toxic.	2	−	−						
17. E. C.	Mild.	4	−	−						

+++ indicates strongly positive reaction, 50 to 70 mm. in diameter, bright red, moderately indurated and tender, of 3 to 4 days duration followed by pigmentation and desquamation; ++, positive reaction, 35 to 50 mm. in diameter, bright red, with slight induration, of 2 to 3 days duration, followed by moderate pigmentation and occasionally slight desquamation; +, moderately positive reaction, 20 to 35 mm. in diameter, red, no induration, of 1 to 2 days duration, with slight pigmentation and no desquamation; and −, no reaction.

cases of scarlet fever. It is furthermore evident from the variations in the intensity of the reactions, that the concentration of the toxic substance varies in different cases. It would also appear by comparison of the positive reactions in Volunteers F. G. B. and E. E. N. on the one hand with those in I. H. and E. McC. (Table II) on the other that individuals vary in their susceptibility. Correlation of the positive and negative reactions with the day of the disease on which the serum was collected is not apparent nor is there a strict correlation between the clinical severity of the cases and the toxic

TABLE III.

Neutralization of Toxic Substance in Scarlet Fever Blood by Normal Human Serum Possessing the Property of Blanching the Rash in Scarlet Fever.

Scarlet fever serum case.	Reactions in Volunteer Test Subject F.G.B.	
	Series A. Unneutralized. 0.3 cc. scarlet fever serum + 0.2 cc. non-blanching serum.	Series B. Neutralized. 0.3 cc. scarlet fever serum + 0.2 cc. blanching serum.
1. A. D.	Strongly positive.	Slightly positive, transient.
2. J. D.	" "	Negative.
3. R. M. A.	" "	Slightly positive, transient.
4. C. M.	Positive.	Negative.
5. N. G.	"	Slightly positive, transient.
6. A. C.	"	Negative.
7. A. Z.	Moderately positive.	"
8. L. W.	" "	"
9. S. S.	" "	"
10. H. C.	" "	"
11. C. P.	" "	"

property of the serums. That the positive reactions were due to a toxic substance specifically related to scarlet fever is indicated by the fact that they occurred only in members of Group A, while all tests in members of Group B were completely negative. Further evidence in support of this view is provided in the following test.

Neutralization of the Toxic Substance in the Blood of Persons with Scarlet Fever by Human Serum Possessing the Property of Blanching the Rash in Scarlet Fever.

IV. The serums from scarlet fever patients (Nos. 1 to 11, Test III), all of which had given positive reactions, were used. Series A: three parts of each serum were

mixed with two parts of normal human serum which had been shown not to blanch the rash in scarlet fever. Series B: three parts of each serum were similarly mixed with two parts of normal human serum which had been shown to blanch the rash in scarlet fever. Both series were incubated in a water bath at 37°C. for 30 minutes. 0.5 cc. of each mixture was injected intracutaneously in an individual of Group A, III (Volunteer F. G. B.) who had been shown to be susceptible to the toxic substance in scarlet fever serum. As shown in Table III all the serum mixtures of Series A gave positive reactions similar to those in Test III, while none of the serum mixtures of Series B gave positive reactions except for a faint transient erythema in Cases 1, 3, and 5.

Hence it may be stated that the toxic substance present in the blood of patients with scarlet fever is readily and completely neutralized by a human serum which produces a positive Schultz-Charlton

TABLE IV.

Neutralization of Toxic Substance in Scarlet Fever Blood by Dochez's Scarlatinal Antistreptococcic Serum.

Scarlet fever serum case.	Reactions in Volunteer Test Subject I.M.W.	
	Series A. Unneutralized. 0.3 cc. scarlet fever serum + 0.02 cc. normal horse serum.	Series B. Neutralized. 0.3 cc. scarlet fever serum + 0.02 cc. antiscarlatinal horse serum.
1. A. D.	Strongly positive.	Negative.
4. C. M.	" "	"
5. N. G.	Positive.	"

rash extinction test. Failure to obtain complete neutralization in three of the eleven tests is not surprising when it is realized that the mixtures contained two unknown variables,—the toxic content of the scarlet fever serum and the antitoxic content of the neutralizing serum.

Neutralization Tests with Dochez's Scarlatinal Antistreptococcic Serum.

Since it had been found by Blake, Trask, and Lynch[14] that Dochez's scarlatinal antistreptococcic serum produces a marked blanching of the rash in scarlet fever similar to the Schultz-Charlton rash extinction phenomenon produced by convalescent scarlet fever serum and by some normal human serums, it seemed probable that Dochez's serum would neutralize the toxic substance in scarlet fever

blood. After a few preliminary trials it soon became evident that a clear-cut result could be obtained only in an individual in whom an intracutaneous injection of normal horse serum produced no immediate or accelerated local serum reaction. Such an individual was finally found and the following test performed.

V. Volunteer I. M. W.; no history of scarlet fever; serum gives a negative blanching test. Scarlet Fever Serums 1, 4, and 5 (Test III) were used. Series A: three parts of each serum were mixed with two parts of a 1:10 saline dilution of normal horse serum. Series B: three parts of each serum were mixed with two parts of a 1:10 saline dilution of Dochez's antiscarlatinal horse serum. Both series were incubated in a water bath at 37°C. for 30 minutes. 0.5 cc. of each mixture was injected intracutaneously in Volunteer I. M. W. As shown in Table IV Series A produced positive reactions, while Series B showed no reactions.

This test not only shows that the toxic substance in scarlet fever blood is readily neutralized by Dochez's scarlatinal antistreptococcic serum, but it also provides evidence which supports the view that Dochez's serum is a specific scarlatinal antitoxic serum. It also suggests that Dochez's serum has a relatively high antitoxic content since 0.02 cc. of the serum completely neutralized 0.3 cc. of Scarlet Fever Serum 1 which was incompletely neutralized by 0.2 cc. of a human blanching serum (IV).

Presence of a Toxic Substance in the Urine of Patients with Scarlet Fever.

Inasmuch as a toxic substance was readily demonstrable in the blood of patients with scarlet fever, it seemed desirable to determine whether a similar substance might be found in the urine. Accordingly the urine of five patients was studied by means of intracutaneous tests in human volunteers.

Since it was to be expected that pseudoreactions from other substances in the urine might obscure the results each test was controlled by a simultaneous neutralization test.

VI. Specimens of urine were collected from five patients with scarlet fever, filtered through Berkefeld filters, and tested for sterility. Parallel series of intracutaneous injections were made in four normal volunteers, three of whom had been shown to be susceptible to the toxic substance in scarlet fever blood, and one of whom served as an insusceptible control. Series A consisted of mixtures of each

TABLE V.

Toxic Substance in Urine of Scarlet Fever Patients.

			Series A. Unneutralized.				Series B. Neutralized.			
Volunteer test subjects			F.G.B.	I.H.	E.McC.	J.D.T.	F.G.B.	I.H.	E.McC.	J.D.T.
Blanching power of serum			0	0	0	+	0	0	0	+
Case.	Day of disease.	Intracutaneous injection.								
1. C. M.	3	Amount of urine.	0.04 cc.	0.03 cc.	0.02 cc.	0.17 cc.*	0.04 cc.	0.03 cc.	0.02 cc.	0.17 cc.*
		Reaction at 48 hrs.	+++	+++	++	−	−	−	−	−
2. N. G.	4	Amount of urine.	0.2 cc.			0.05 cc.†	0.2 cc.			
		Reaction at 48 hrs.	++			−	−			
3. L. V.‡	4	Amount of urine.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.
		Reaction at 48 hrs.	−	−	−	−	−	−	−	−
4. C. R.‡	2	Amount of urine.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.
		Reaction at 48 hrs.	−	−	−	−	−	−	−	−
5. F. N.	3	Amount of urine.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.
		Reaction at 48 hrs.	−	−	−	−	−	−	−	−

* The ratio of urine to serum in this test was 5:1.

† The urine was not mixed with serum in this test.

‡ 0.2 cc. of undiluted urine was tested in a susceptible subject (Volunteer I. M. W.) and gave a negative result.

urine with normal human serum which failed to blanch the rash of patients with scarlet fever. Series B consisted of similar mixtures of each urine with normal human serum which blanched the rash in scarlet fever. The ratio of urine to serum in the mixtures was 1:9 except in one instance (Case N. G.) in which it was 1:1. Both series were incubated in a water bath at 37°C. for 30 minutes prior to injection. The actual amount of urine in the urine-serum mixtures injected varied from 0.02 to 0.2 cc. Reactions in Series A were considered positive only when they persisted for 48 hours or more and were followed by pigmentation or desquamation with negative reactions in Series B and in the insusceptible control at 48 hours. Moderate transient erythema of about 24 hours duration occurred with nearly all injections and was disregarded. As shown in Table V, two of the five cases, both of which had given positive reactions with their serums, gave strongly positive reactions in Series A with negative reactions in the controls. Three were negative in both series. The positive reactions were characterized by bright red erythema, 40 to 60 mm. in diameter, moderate induration and tenderness, were of 3 to 4 days duration, and were followed by pigmentation and desquamation.

The foregoing data show that a toxic substance can be demonstrated in the urine of some cases of scarlet fever. This substance is neutralized by human serum that blanches the rash of scarlet fever but not by human serum that fails to blanch the rash. It causes a well defined local reaction in the skin of individuals whose serums give a negative Schultz-Charlton rash extinction test, no reaction in persons whose serums give a positive rash extinction test. In these respects it is similar to the toxic substance found in the blood of scarlet fever patients. In the tests exceedingly small amounts of unconcentrated urine were employed in order to avoid confusion with false reactions. It seems probable that the toxic substance specifically related to scarlet fever will be found in most scarlet fever urines when a more ready method for its detection than that of intracutaneous tests in human beings is devised.

Daily Observations on the Presence of a Toxic Substance in the Blood and Urine of a Patient with Scarlet Fever.

The data so far reported have dealt with single observations on a series of patients. From the results obtained it has not been possible definitely to relate the presence or absence of the toxic substance in the blood and urine with the course of the disease. Furthermore, although there is in general a satisfactory agreement between the

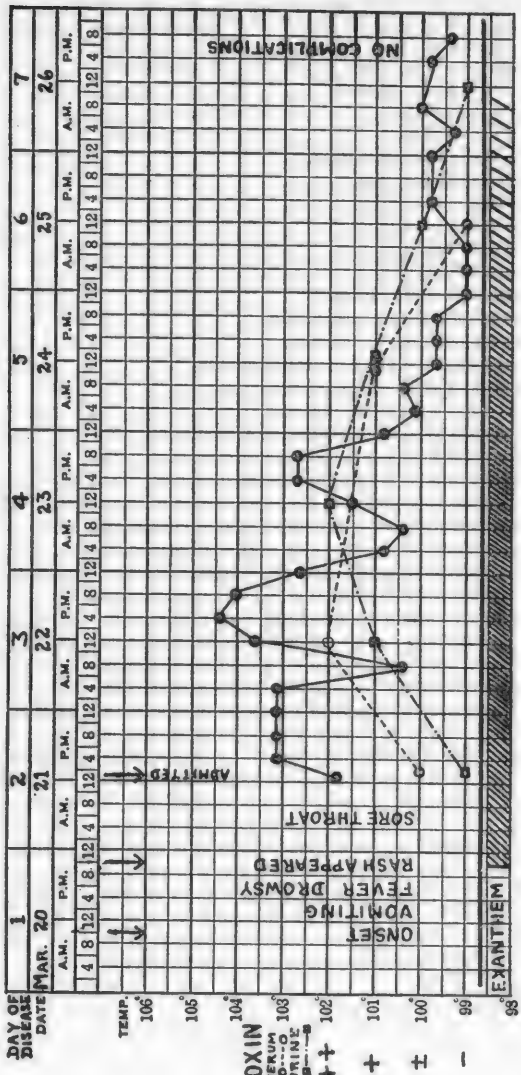

CHART 1. Daily observations on toxin in blood and urine of scarlet fever, Case N. G.

clinical severity of a case and the presence or absence of the toxic substance in the blood and urine, there are certain exceptions as in Cases J. M. (II), J. D., and M. D. (III). It seemed desirable, therefore, to make daily observations on a series of cases in order to obtain more definite information concerning these points. One study of this kind has been completed and may properly be included here.

VII. Case N. G., male; age 6. Admitted to the hospital on the 2nd day of scarlet fever, about 27 hours after onset. Moderately severe. Samples of blood and urine were collected daily throughout the disease. These were tested by intracutaneous injections in susceptible human volunteers, with the appropriate insusceptible volunteer controls and control neutralization tests. 0.3 cc. of serum and 0.2 cc. of urine were used in the tests. Toxic substance was present in the blood from the 2nd to the 5th day, in the urine from the 3rd to the 6th day. The relation of its concentration as roughly measured by the severity of the local reactions in the test subjects to the course of the disease is shown in Chart 1.

SUMMARY.

A series of observations on the blood of patients acutely ill with scarlet fever has shown that a toxic substance can be demonstrated in the serum by means of intracutaneous injections of the serum in persons who have not had scarlet fever and whose serums fail to blanch the rash in scarlet fever. The reaction caused by this substance consists of a bright red local erythema, varying from 20 to 70 mm. in diameter, of 1 to 4 days duration. The severer reactions are moderately indurated and tender, and are followed by pigmentation and desquamation. Control injections in persons whose serums blanch the rash in scarlet fever cause no reaction. The toxic substance is not neutralized by mixture with a human serum which gives a negative blanching test but is readily neutralized by a human serum which gives a positive blanching test. It is not neutralized by normal horse serum, but is completely neutralized by Dochez's scarlatinal antistreptococcic serum.

In a limited number of observations on the urine of patients with scarlet fever a similar toxic substance has been found in two out of five cases studied.

Since the toxic substance described appears to resemble the toxic substance found in the filtrates of scarlatinal hemolytic streptococcus

cultures by Dick and Dick and since it is neutralized not only by a blanching human serum but also by Dochez's scarlatinal antistreptococcic horse serum, the experiments reported support the conception that scarlet fever is a local infection of the throat by a particular type of *Streptococcus hæmolyticus* capable of producing a toxin which is absorbed and is the cause of the general manifestations of the disease.

EXPLANATION OF PLATE 16.

FIG. 1. Volunteer A. P. Positive local reaction on right forearm at site of intracutaneous injection of Serum A from scarlet fever case, M. R., 3rd day of disease. 26 hours after injection.

FIG. 2. Volunteer A. P. Site of control injection, left forearm, Serum B from scarlet fever case, M. R., 21st day of convalescence. 26 hours after injection.

FIG. 1.

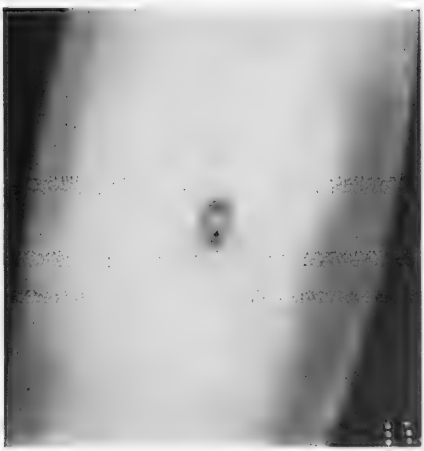

FIG. 2.

(Trask and Blake: Toxic substance in scarlet fever.)

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE.

V. The Effect of Diet on Host Resistance.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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One of us (Webster) has shown that the general non-specific resistance of mice bred at the Rockefeller Institute includes important elements associated with heredity and that, by selective breeding, the general resistance of a mouse population may be altered.[1] We wish now to describe some experiments which demonstrate the effect of diet on host susceptibility to infection and intoxication.

For some years the mice in the breeding room of the Rockefeller Institute have received a diet consisting of a daily ration of baker's bread soaked in fresh pasteurized Grade B milk warmed to at least 60–70°C., supplemented by two weekly feedings of an oatmeal and buckwheat mixture and one weekly feeding of dog biscuit. On this diet the animals thrive and breed well and are to all appearances in excellent health.

It was thought desirable to compare the resistance of these mice with that of similar mice raised on some other stock diet in general use. Consequently, a so called normal diet for mice and rats, advocated by E. V. McCollum, was chosen[2] which consists of the following ingredients: wheat 67.5 per cent, casein 15 per cent, milk powder 10 per cent, NaCl 1 per cent, $CaCO_3$ 1.5 per cent, and butter fat 5 per cent. This diet was prepared by mixing the finely ground whole wheat with the inorganic salts, casein, commercial milk powder, and the clear fat from melted sweet butter. Small quantities were continually avail-

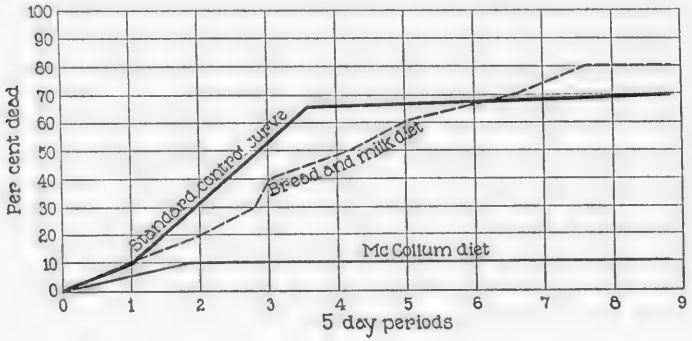
[1] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 879.

[2] This particular formula for the McCollum complete diet was obtained through a personal communication from Dr. E. V. McCollum.

able to the mice in porcelain salve jars. A constant supply of fresh water was also provided.

Experiment 1 was planned to compare the resistance of mice fed on the bread and milk diet with that of similar mice fed on the McCollum diet.

Experiment 1.—Twelve young female mice, bred at the Rockefeller Institute and in early pregnancy, were chosen and caged in separate boxes in the breeding room. Six of these mice were maintained on the regular bread and milk diet and six were changed to the McCollum diet prepared from vitamine-free casein and purified butter fat.[3] The litters of the first group were therefore nourished by McCollum diet mothers while those of the second group were fed by bread and milk diet mothers. After weaning time, the respective diets were continued, the



TEXT-FIG. 1.

first group, ten mice, receiving McCollum diet and the second group, ten mice, the Institute bread and milk diet throughout the entire experiment.

When the young mice had reached a weight of 16 to 18 gm. each, they were placed in separate jars and inoculated by stomach tube with 4,500,000 mouse typhoid bacilli, Type II,[4] grown in plain broth pH 7.4 for 16 hours. Autopsies were performed on those dying and cultures taken from heart's blood and intestinal contents. Mortality curves for the two groups were plotted, together with the standard control curve constructed by Webster[5] for Rockefeller Institute mice fed on the regular breeding room diet (Text-fig. 1.).

Experiment 2.—The twelve females described in Experiment 1 were kept on their respective diets and mated a second time. The F_1 generation was fed and

[3] These materials were obtained through the kindness of Dr. P. A. Levene and his department.

[4] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

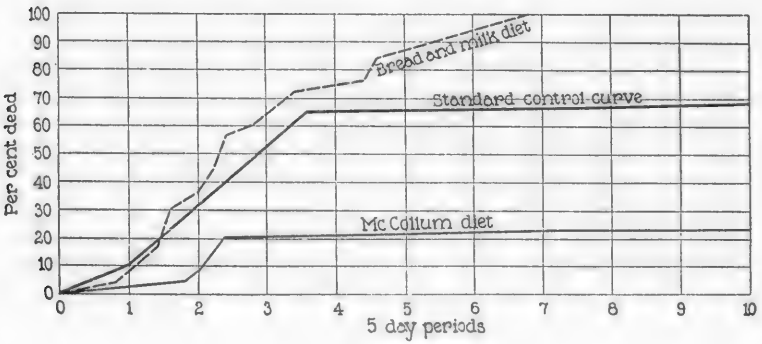
[5] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 231.

treated as in Experiment 1. One group, twenty-six mice, continued to receive McCollum diet after weaning and the second group, twenty-six mice, was fed the bread and milk diet.

When the young had reached a weight of 16 to 18 gm. each, they were injected as before with a dose of 3,600,000 bacilli and caged separately for observation. The respective diets were continued; fatal cases were autopsied and cultured for the mouse typhoid bacilli. Mortality curves of the two groups of mice, together with the standard control curve for Institute mice, are plotted in Text-fig. 2.

The results of these two experiments show that mice fed on the McCollum diet are more resistant to mouse typhoid infection than are similar mice nourished on a bread and milk diet.

Experiment 3.—The experiment was repeated a third time under somewhat different conditions. The pregnant females from the Rockefeller Institute stock,

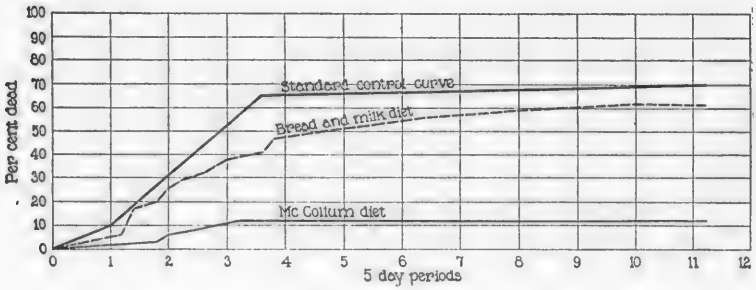


TEXT-FIG. 2.

instead of being kept in the breeding room under the care of the regular attendant, were cared for by us in another room. A great effort was made to have this care in every way comparable to the care given the mice in the breeding room. The pregnant females were again divided into two diet groups, one receiving the regular breeding room diet, the other a McCollum complete diet, the latter prepared from commercial materials instead of the carefully purified materials used in Experiments 1 and 2. The small mice were weaned when 3 weeks old, and were all continued on the same diet that their mothers had received during pregnancy, and lactation. When they were 8 to 10 weeks old, thirty-three from the McCollum diet series and thirty-six from the control diet series, all weighing at least 18 gm., were assembled in separate battery jars and were each given by stomach tube 0.005 cc. of a 24 hour broth culture of M. T. II (7,000,000 organisms) in a 0.5 cc. volume. They were observed through the following 8 weeks, the deaths charted and cultures taken at autopsy from the spleen and feces, to determine the presence of M. T. II. The results of the experiment are presented graphically in Text-fig. 3.

Text-fig. 3 shows the standard control curve for the mice from the breeding room,[5] the curve for the control diet series described above, and the curve for the corresponding McCollum diet series. It will be seen that the curve for the control diet series runs somewhat lower than the standard control curve. Nevertheless, it is obvious that in this experiment, as well as in Experiments 1 and 2, the mice fed on the McCollum complete diet were far more resistant to the infection than were those fed on our regular breeding room diet, despite the fact that before the experiment was begun the control diet mice were to all appearances as healthy as were those on the McCollum diet.

One of us[6] has shown that if a group of mice from the Rockefeller Institute breeding room is given *per os* a sufficient dose of bichloride of mercury a mortality curve can be obtained which resembles the



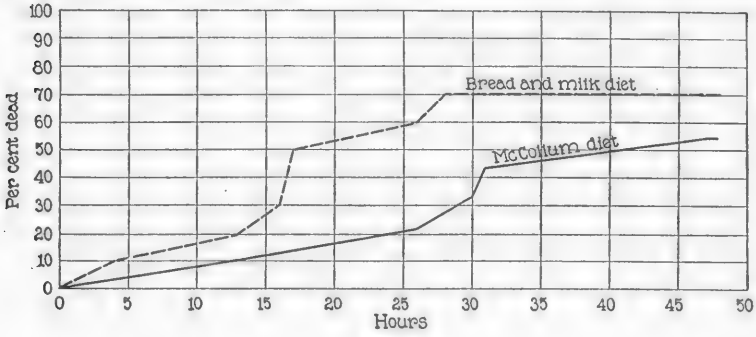
TEXT-FIG. 3.

curves obtained with fixed doses of M. T. II. It has further been shown that the resistance to mercury bichloride of a mouse population that has survived infection with M. T. II is greater than that of normal mice,[7] indicating that the resistance of these individuals is to some extent non-specific. Since we had observed that the resistance of our mice to mouse typhoid infection can be modified through diet, we wished to know whether under similar circumstances they would also prove to be more resistant to bichloride of mercury given by mouth. Accordingly, a small number of the mice described above were used in an experiment with bichloride of mercury. For such a test it is essential that mice of very nearly equal weight be used.

[6] Webster, L. T, *J. Exp. Med.*, 1923, xxxviii, 45.
[7] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 129.

Experiment 4.—Ten mice were selected from the control diet series and nine from the McCollum diet series, all weighing 18 to 19 gm. Each of these mice received, by stomach tube, 0.5 cc. of a 1:200 dilution of bichloride of mercury in distilled water (0.0025 gm.), individuals of the two series receiving it alternately. The mice were observed over a period of 48 hours, deaths being recorded hourly. The results are shown graphically in Text-fig. 4.

It will be seen from Text-fig. 4 that the mice on the McCollum diet were distinctly more resistant to the injection of bichloride of mercury than were those on the control diet. The McCollum diet curve is very much more gradual and the final mortality rate of these mice is 15 per cent below that of the mice on the control diet. It is to be regretted that at the time this experiment was performed larger numbers of mice on the two diets were not available. Nevertheless,



TEXT-FIG. 4.

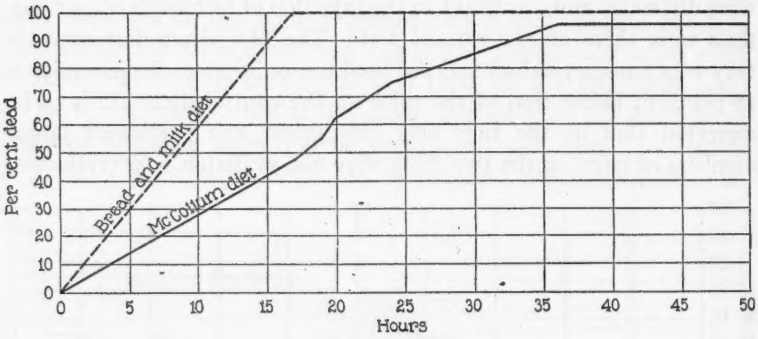
when taken in conjunction with the three experiments on the effect of diet upon mouse typhoid infection, the results obtained in Experiment 4 would seem to be significant.

Additional evidence in support of the non-specific nature of the resistance conferred by the McCollum diet was obtained from an experiment kindly made for us by Dr. J. Bronfenbrenner in which he compared the resistance of McCollum diet-fed mice and bread and milk diet-fed mice to *botulinus* toxin.

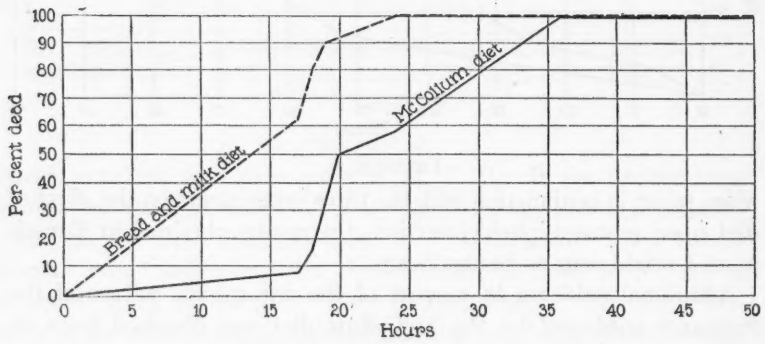
Experiment 5.—Although the mice used in this experiment were the survivors from Experiment 3 and might therefore be considered as a selected group, both the McCollum diet series and the bread and milk diet series had undergone the same treatment and were therefore suitable for further investigation. Fifteen McCollum diet mice and seven bread and milk diet mice received 1 M.L.D. of *botulinus*

toxin intraperitoneally and twelve McCollum diet mice and eight bread and milk diet mice received 2 M.L.D. intraperitoneally. The percentage mortality in the two groups of mice is plotted in Text-figs. 5 and 6.

The curves (Text-figs. 5 and 6) indicate that the mice fed on the McCollum diet were distinctly more resistant to *botulinus* toxin than were similar mice maintained on a bread and milk diet. Although



TEXT-FIG. 5.



TEXT-FIG. 6.

the final mortality is the same for each group, the lag, as in the experiment with mercury bichloride, is far greater among the mice fed on the McCollum diet than among those fed on a bread and milk diet.

This experiment, taken in conjunction with Experiment 4 and the previous work reported, constitutes further evidence that the so called general resistance of the host may be largely non-specific in nature.

DISCUSSION.

The literature relating to the effects of diets on health and disease contains few specific facts of an experimental nature. However, various investigators in the relatively new field of quantitative dietetics have expressed the opinion that the response of animals to inoculation with bacteria and certain poisons is affected by dietary deficiencies in salt, vitamine, protein, and other food substances.

We have examined two sets of mice of the same race and stock from the Rockefeller Institute breeding room as regards their response to a strain of mouse typhoid bacilli, mercury bichloride, and *botulinus* toxin. One set was from the general stock which had been fed on the regular breeding room diet, as given above, for many generations without any evidence of apparent deficiency. The second set of mice, also from the regular Institute stock, differed only in being fed on McCollum's diet. These mice throve well, of course, but not perceptibly better than the first set. And yet the far greater resistance of the McCollum diet-fed animals both to *Bacillus pestis caviæ*, *botulinus* toxin, and mercury bichloride is striking.

To explain this disparity on the basis of dietary effects in the nature of deficiencies is to make assumptions on factors that are very subtle. It is true that such subtle deficiencies are postulated by McCollum[8] and Cramer.[9] Possibly the future may reveal the existence of a deficiency operating insidiously and over a long period of time through many generations of animals. Until such time as an undoubted experimental basis is secured for this conception, we prefer merely to record our experimental results without discussing their ultimate meaning and explanation. The fact is, however, indisputable that profoundly striking effects on the behavior of mice to an infectious bacterium, a bacterial toxin, and a chemical poison can be produced by feeding, in the absence of any manifestations that are attributable to dietary deficiencies as now recognized.

[8] McCollum, E. V., in Nelson Loose-leaf medicine, New York, Edinburgh, London, Paris, and Toronto, 1920, vii, 162.

[9] Cramer, W., *Brit. J. Exp. Path.*, 1922, iii, 298.

CONCLUSIONS.

White mice from the Rockefeller Institute breeding room fed on a McCollum complete diet, consisting of whole wheat (67.5 per cent), casein (15 per cent), milk powder (10 per cent), NaCl (1 per cent), $CaCO_3$(1.5 per cent), and butter fat (5 per cent) are more resistant to mouse typhoid infection, mercury bichloride intoxication, and *botulinus* toxin than are similar mice fed on bread and pasteurized milk supplemented by an oatmeal and buckwheat mixture and dog biscuit.